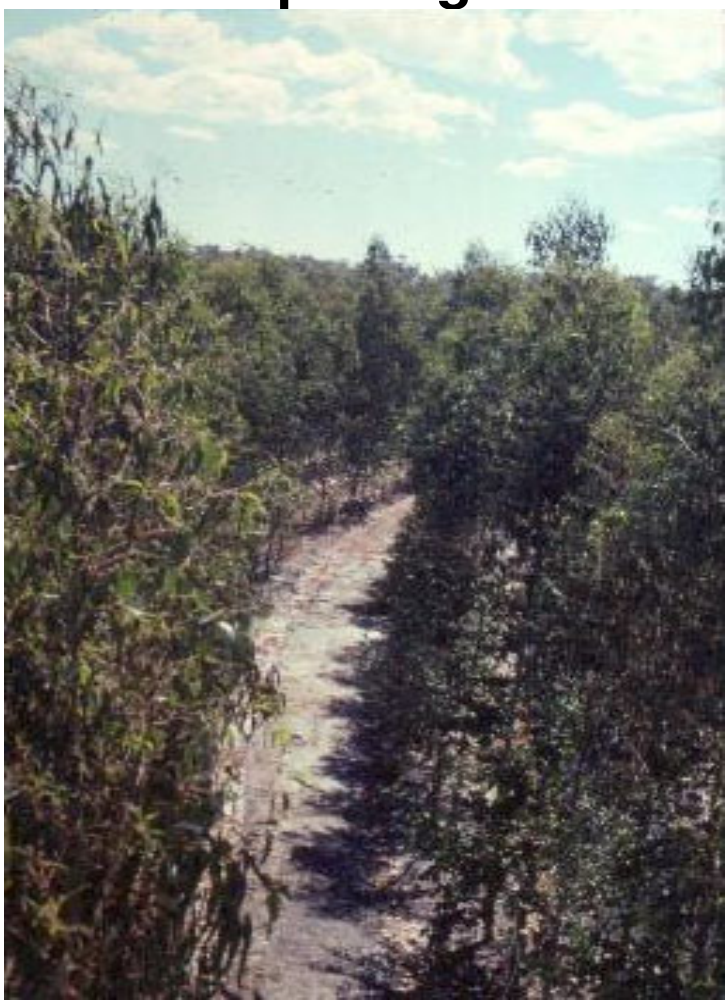
Occurrence and variation of *Endothiella eucalypti* in *Eucalyptus globulus* plantations of south-western Australia and the influence of some biotic and abiotic factors on the response of the host to the pathogen.



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Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

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Abstract

As the *Eucalyptus globulus* plantation industry expands and matures in southwestern Australia (WA), the impact of disease within the plantation environment is predicted to increase. This thesis investigated the most abundant canker-causing pathogen associated with branch and stem cankers, *Endothiella eucalypti* the anamorph of *Cryphonectria eucalypti*. *Endothiella eucalypti* was widespread, although at low incidence, throughout the WA plantation estate and was frequently observed sporulating on the bark of healthy hosts in the absence of disease. Regions with a long (approximately 20 years) plantation history, such as Bunbury, had the highest incidence of this pathogen. A high degree of variability in pathogenicity, growth rate and colony morphology was observed between WA isolates of *En. eucalypti*.

In the glasshouse, a significant variation in susceptibility of seven *E. globulus* provenances to *En. eucalypti* was observed. Although an interaction between the *E. globulus* provenance and *En. eucalypti* isolate was recorded, some provenances were generally more susceptible than others. In two 18-month-old plantations, the susceptibility of three provenances to *En. eucalypti* was significantly influenced by environmental conditions. Visual assessment of general tree health indicated that less healthy trees had smaller lesions than healthy trees. It is hypothesised that the selection of *E. globulus* provenances to suit site conditions in the future should decrease the risk of serious disease, especially on marginal sites.

Endothiella eucalypti caused disease in intact stems of two-year-old *E. globulus* under glasshouse conditions. This suggests that *En. eucalypti* may not require a wound to infect in the field.

Vegetative compatibility groupings between WA *En. eucalypti* isolates indicated a relatively high degree of genotypic diversity within the WA asexual population of *En. eucalypti*, whereas inter-simple sequence repeats PCR (ISSR-PCR) analysis indicated a lower level of genotypic diversity. Discrepancies between traditional and molecular techniques, such as ISSR-PCR, was attributed to the more specific gene-to-gene analysis afforded by molecular techniques. ISSR-PCR successfully distinguished variability within the *En. eucalypti* population and with the teleomorph, isolated in South Africa. It also separated *Cryphonectria cubensis* isolates from the *C. eucalypti* isolates.

As copper is the micronutrient most limiting growth of *E. globulus* in WA, its role in the resistance of two *E. globulus* provenances was examined in a glasshouse trial. Lesion extension or defence responses of *E. globulus* to *En. eucalypti* did not differ between Cu-adequate and Cu-deficient plants. It is suggested that constitutive levels of host defence enzymes played a more important role in providing protection for the host against *En. eucalypti* than the external supply of copper.

A reduction in the canopy volume of *E. globulus* within plantations due to insect herbivory or foliar pathogens, such as *Mycosphaerella* spp., has been reported to predispose the host to disease caused by non-aggressive canker-causing fungi. Under two separate glasshouse trials, conditions of 100% defoliation and 80% defoliation maintained over six weeks prior to inoculation, were required to significantly increase lesion extension caused by *En. eucalypti* in *E. globulus* stems. The ability of defoliated *E. globulus* to retain a degree of resistance to *En. eucalypti* was attributed to the rapid replacement of foliage and up-regulation of photosynthesis in remaining leaves. The carbohydrate reserves of the plant were depleted following defoliation and remained depressed regardless of the length of time the trees remained defoliated.

In conclusion, the endophytic habit of *En. eucalypti* poses a threat to highly stressed trees, however it does not appear to be an immediate threat to WA plantation health. Although *En. eucalypti* has not yet been responsible for a major disease outbreak in WA, the impact of this disease on plantation-grown eucalypts elsewhere in Australia and worldwide serves as an indication of its potential to affect WA plantations.

Table of Contents

Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
CHV	Cryphonectria hypovirus
CI	consistency index
CRY	*Cryphonectria cubensis*
Cu^+	fertiliser treatment with copper
Cu^-	fertiliser treatment without copper
DCLM	Department of Conservation and Land Management
DDI	double de-ionised
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EDTA	ethylenediamine tetracetic acid
FW	fresh weight
G	genotypic diversity
IBDU	isobutylidene diurea
ICPAES	inductively-coupled plasma atomic emission spectrometry
ISSR	inter-simple sequence repeats
ITC	Integrated Tree Cropping Ltd
LSD	Least significant difference
MLD	*Mycosphaerella* leaf disease
NA	not available
NB	no bands
P	phosphate
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDA+S	potato dextrose agar containing streptomycin sulfate
PO	peroxidase
PVC	polyvinylchloride
PYC	pycnidia
RAMS	randomly amplified microsatellites
RFLP	restriction fragment length polymorphism
RI	retention index
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SE	standard error
SOD	superoxide dismutase
SSR	single sequence repeats
T	transect
VC	vegetative compatibility
WA	Western Australia
YFEL	youngest fully expanded leaf
YML	young mature leaves

Chapter 1

Literature Review

Hardwood plantation forestry is a new and expanding industry in southwestern Australia, with *Eucalyptus globulus* (Tasmanian bluegum) plantations being established in monoculture predominantly on agricultural land. Increasing value is placed on plantation-grown timber products as well as pulpwood, to reduce the need for harvesting native timber stands. In Australia, it is estimated that three million hectares of hardwood plantations will be in cultivation by 2020, of which more than two-thirds are expected to be *E. globulus* (National Forest Inventory, 2002). *Eucalyptus globulus* is a valuable forestry species as it has a rapid growth rate, high-quality wood fibre and is tolerant to a large range of environmental conditions (Strauss, 2001). However, sustainable production from plantations will only be possible if (i) environmental stresses can be kept to a minimum and (ii) major pest and diseases can be avoided or their impact on tree growth and wood quality kept below economically significant levels.

The *E. globulus* industry is relatively young in Australia and most plantations are still in their first rotation. First-rotation growth rates are high, partly because of previous use of fertilizers and annual leguminous pasture species by farmers. However, over the last five years, the levels of damage due to foliar and stem diseases, insect attack and environmental stress have increased in *E. globulus* plantations in southwestern Australia (Maxwell *et al.*, 1998). As the industry matures there is a need to better understand the factors which affect plantation health, such as environmental stresses, the impact of pests and disease, and the interaction of these factors, in order to ensure an economically sustainable industry for the future.

Eucalyptus globulus

Eucalyptus globulus (Labill. 1799), was one of the earliest eucalypts to be validly named and brought into cultivation (Eldridge *et al.*, 1993). It is native to southeastern Australia (natural range is approximately 38°26'S to 43°30'S (Eldridge *et al.*, 1993), occurs in a number of different environments and is likely to have had a complex history of environmental changes, migration, barriers to dispersal and intergradation with related subspecies (Dutkowski and Potts, 1999). More specifically, it occurs in natural forest ecosystems of eastern Tasmania in populations along the coast and up to

20 km inland. Here it grows in relatively dry open forest where mean annual rainfall can be as low as 550 mm. *Eucalyptus globulus* also extends 60 km inland, at more than 500 m above sea level (latitude 43°S), where rainfall is more than 1000 mm and trees grow in closed forests to a height of 70 to 80 meters. In addition, several small stands of *E. globulus* are located along the wet west coast of Tasmania. *Eucalyptus globulus* occurs on several of the Bass Strait Islands and in southern Victoria on the southern end of the mainland at Wilsons Promontory and on the coastal side of both the Otway and Strzelecki Ranges (Figure 1.1A) (Eldridge *et al.*, 1993).

Eucalyptus globulus is a medium-sized to tall forest tree which develops different juvenile, transitional and adult leaf forms during successive life stages (Figure 1.1C). The broad, thin, blue-grey, glaucous, dorsiventral and hypostomatal juvenile leaves are succeeded by narrow, thick, green, isobilateral and amphistomatal leaves with the development of the tree (James and Bell, 2000). *Eucalyptus globulus* exhibit solitary, usually sessile flowers with pedicels usually absent and flower between September and December (Brooker and Kleinig, 1983).

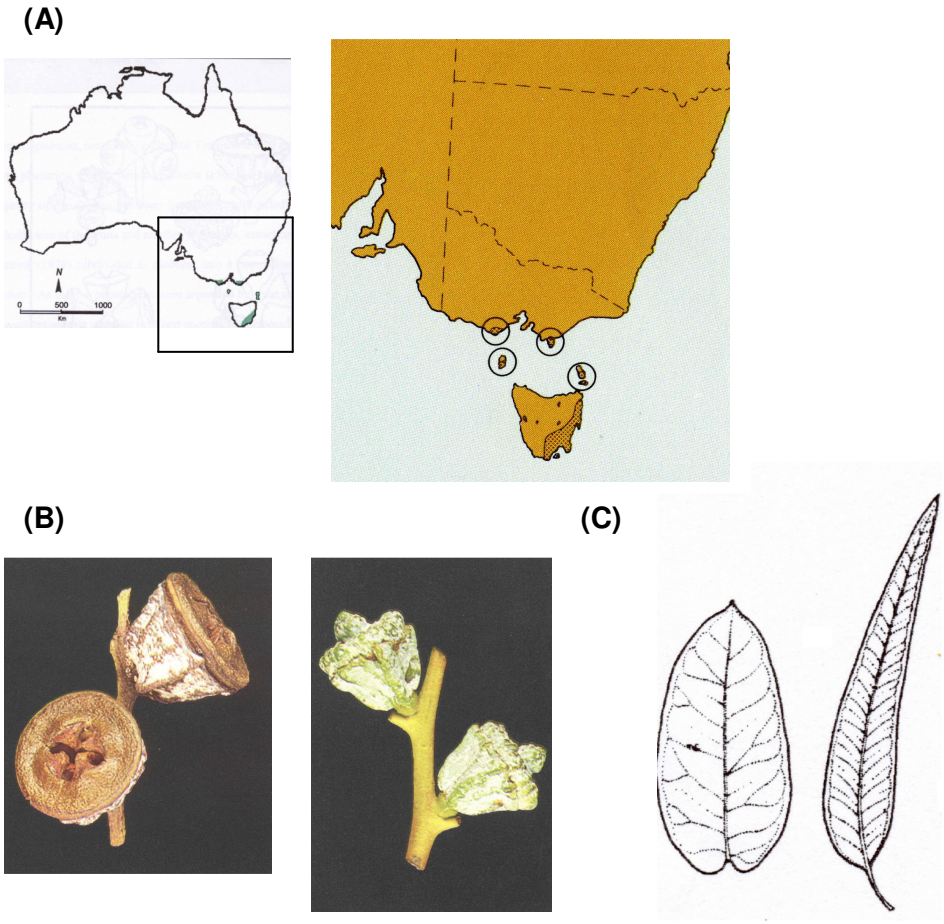


Figure 1.1 (A) Natural distribution of *Eucalyptus globulus* in Australia; (B) fruits of *E. globulus*; (C) juvenile (left) and mature (right) leaf stages of *E. globulus.* Figure adapted from Walsh and Entwisle (1993), Brooker and Kleinig, (1983).

Eucalyptus globulus plantations

The species *E. globulus* is subdivided into four distinctly different subspecies (Walsh and Entwistle1993; Chippendale, 1988). *Eucalyptus globulus* plantations are primarily composed of *E. globulus* subsp. *globulus*, however throughout this thesis it shall be referred to as *E. globulus,* unless otherwise noted.

Eucalyptus globulus was the first eucalypt species to be used outside Australia as an ornamental and plantation tree (Eldridge *et al.*, 1993). It responds well to intensive silviculture and achieves high growth rates under favourable conditions (Judd *et al*., 1996). There are extensive plantations of *E. globulus* in Portugal, Spain and Chile and smaller areas in Bolivia, China, Columbia, Ethiopia, Argentina, Peru, the USA (California), and several other countries (Eldridge *et al.*, 1993). *Eucalyptus globulus* was one of the most widespread plantation pulpwood species in the world with over 1 700 000 ha planted by 1996 (Dutkowski and Potts, 1999). The wood is used primarily for pulpwood, firewood and mine timber.

Increased demand for hardwood pulp has led to a rapid expansion in eucalypt plantings in Australia (Judd *et al*., 1996). Significant areas of eucalypt plantations have been established on lowland sites in eastern Victoria, northern Tasmania and the southwest of Western Australia (WA), with *E. globulus* the most widely planted eucalypt species (Bennett *et al.*, 1997; Judd *et al*., 1996; Eldridge *et al.*, 1993). *Eucalyptus globulus* is currently the dominant hardwood plantation resource in Australia (National Forest Inventory, 2002).

Eucalyptus globulus plantations

Eucalyptus globulus was first commercially planted in WA in 1980 and has since become the major hardwood plantation species for mine timber, wood chips and land rehabilitation (Breidahl, 1999; Eldridge *et al*, 1993;). It was selected for initial and subsequent plantings as it (i) had been grown successfully in Mediterranean climates; (ii) possessed desirable pulping qualities; (iii) was resistant to *Phytophthora cinnamomi*; (iv) could coppice from the stump and (v) had potential for other uses such as sawn timer and veneer (Breidahl, 1999).

History of Eucalyptus globulus *plantations in brief*

Between 1980 and 1985 establishment of commercial plantations in WA was slow with less than 200 ha planted per annum. However, the benefits of establishing plantations on ex-pasture sites became obvious and the rate of hardwood planting increased

steadily from the early 1990's (National Forest Inventory, 2002). Another factor in the increased planting was the initiation of the Department of Conservation and Land Management's Tree Trust Program in 1988 to 1989, which led to the establishment of 5 000 hectares. From the period of 1991 to 1995, annual plantings increased from 2 000 hectares per annum to 10 000 hectares per annum. Commercial harvesting operations commenced during this period with an initial export of 30 000 tonnes of woodchips to Japan in 1994. However, a major drought during the summer and autumn of 1993/94 resulted in widespread deaths of *E. globulus* in 3-6 year-old plantations on sites which were shallow to bedrock or had impenetrable hardpans. This led to routine drilling of all potential sites to a depth of 2.1 meters during site assessment.

Between 1996 and 1999, plantings continued to increase, with more than 20 000 hectares established per annum. During this time, annual woodchip exports to Japan increased from 50 000 to 100 000 tonnes (Breidahl, 1999). In 1999, with 120 000 hectares of *E. globulus* plantations in WA, it was estimated that by 2007 the level of wood production would increase from 100 000 tonnes/ annum to more than 5 million tonnes/ annum (Breidahl, 1999). By 2001, WA had the largest area of hardwood plantations in Australia with 234 700 hectares (or 40% of national area) (National Forest Inventory, 2002). However, between 2000 and 2001, the rate of establishment of new hardwood plantations (first rotation) decreased significantly in WA (by 68%) and Australia-wide. This decline was not unexpected and was attributed to a change in tax laws (National Forest Inventory, 2002).

The Australian forest plantation estate continued to expand in 2002 with 12 754 hectares of hardwood established in WA. Although this area was greater than in any other state, it was 30% less than the 2001 levels. As of December 2002, the total area of hardwood plantations in Australia was 638 300 hectares, of which 247 500 hectares (39%) were planted in WA (National Forest Inventory, 2003). At present, ninety percent of the new plantations established in Australia are comprised of hardwood species. However, the extended drought of 2002-3 in many regions, had a significant impact on plantation establishment throughout Australia (National Forest Inventory, 2003). Therefore, if the vision for Australian forestry is to reach the goal of three million hectares in cultivation by 2020, investment will have to occur in different regions, perhaps using different species than those that have dominated new plantings in the last ten years (National Forest Inventory, 2003).

In southwestern Australia, plantations have been established on land that has been previously used for agriculture (White *et al.*, 1999; Aggangan *et al.*, 1998) across a range of soil types and rainfall gradients (700 to 1500 mm). Generally the soils of this region have a low level of natural fertility as the parent materials from which they have been formed have been subject to extensive periods of weathering and laterisation (Aggangan *et al.*, 1998; Bennett *et al.*, 1997). Nitrogen and phosphorus are frequently the major nutrients limiting plant growth (Aggangan *et al.*, 1998). Although eucalypts are native to Australia, the species used in plantations such as *E. globulus* plantations in WA, are often not endemic to the region in which they are planted. There is also increased disease risk in the plantation environment as genetic variation is reduced, even though clonal forestry is not yet practised. At present, management is aimed at maximizing growth which means limiting stresses such as drought and damage caused by disease.

Climatic conditions in southwestern Australia

Southwestern Australia experiences a Mediterranean climate (Havel, 1975) with a strong summer/winter pattern in temperature and rainfall. Rainfall is usually confined to the winter months (June to August), with minimal rainfall during summer (December to February). However, substantial summer rainfall events have been recorded (Lucas, 2003). Rainfall generally decreases inland from the coast of southwestern Australia.

Growth requirements for Eucalyptus globulus

The basic growing requirements for *E. globulus* are a maximum of seven months dry season, mean maximum temperature (hottest month) of 19 °C to 30 °C and mean minimum (coldest month) of 2 °C to 12 °C. Optimal *E. globulus* growth and development is achieved in deep, sandy clay soils, however good growth is also achieved on clay-loams and clay soils providing the soils are well drained. Limiting factors associated with soil types include insufficient soil depth, poor drainage and salinity (Eldridge *et al.*, 1993).

As a plantation species, *E. globulus* is considered relatively adaptable to its soil requirements if grown under short rotation and favourable climatic conditions (Poynton, 1979). *Eucalyptus globulus* requires a minimum rainfall of 600 mm per annum in regions with a Mediterranean climate, such as WA. They do grow in regions with rainfall of less than 600 mm, however the trees are prone to drought stress (White *et al.*, 1999). Therefore, *E. globulus* plantations are located in areas of mild climate, free from

severe frosts and without severe drought. Plantations are cut on a coppice rotation of about 8 to 12 years, usually two or three times (Eldridge *et al.*, 1993). *Eucalyptus globulus* grown for pulp and sawn timber have an average of 10 and 25 years, respectively (Dell *pers. comm.*).

The growth of many eucalypt species in plantations can be increased across a range of soil types and climates, by the addition of fertilisers. For example, at planting, and in the early stages of tree establishment, *E. globulus* seedlings require nitrogen and phosphorus fertilisers to ensure early growth and high survival rates (Bennett *et al.*, 1997). The requirement for nitrogen and phosphorus is often less or unnecessary on ex-farm sites, with a history of fertiliser use. However, additions of these macronutrients in forms low in micronutrients, such as copper, result in the development of micronutrient deficiencies (Dell, 1994; Turnbull *et al.*, 1994). Micronutrient deficiencies impact on the growth rate and overall structure of the tree, reducing resistance to pathogens and the quality of the timber (Gherardi *et al.*, 1999; Dell, 1994). As the area of eucalypt plantations increases, the range of site conditions also increases and the response of eucalypts to fertilisers will become increasing important (Judd *et al.,* 1996). Therefore, correct fertiliser additions should be/are an integral part of establishment practices for eucalypt plantations in Australia (Bennett *et al.*, 1997). Good nutrition is one site factor which can be managed effectively to ensure improved productivity (Hooda and Weston, 1999).

Major diseases associated with eucalypts

Although the environment in the lower southwest of WA is suitable for growth of *E. globulus* plantations, problems with disease, arising from a monoculture system are increasing as the industry ages. Experience from plantations in other parts of the world (Roux *et al*., 2000; Heather and Griffin, 1984), has shown that disease can severely affect the productivity of *E. globulus* plantations. For example, damage to the leaves of *E. globulus* caused by *Mycosphaerella* led to the discontinued use of this species in South African plantations during the early 1930's (Crous, 1998; Lindquist, 1987). Therefore, careful monitoring of disease levels, good management practices and selective breeding of more resistant *E. globulus* provenances is required to ensure that the devastation seen in South Africa and elsewhere (e.g.; Brazil, Chile) is not repeated in WA. This thesis focuses on the major diseases which have the potential to impact *E. globulus* in WA.

The major fungal pathogens with the potential to affect *E. globulus* plantations in WA can be divided into leaf and stem diseases. This thesis focuses on stem diseases affecting plantation-grown *E. globulus,* however leaf diseases are discussed briefly as they may have the potential to increase the susceptibility of *E. globulus* to stem diseases.

Leaf diseases

A study undertaken by Davison (1995) identified *Mycosphaerella* spp., *Cylindrocladium* spp., *Aulographina eucalypti* (Cooke and Massee) von Arx and Muller and *Puccinia psidii* Winter as the major leaf pathogens with the potential to affect *E. globulus* in plantations in WA. *Cylindrocladium* spp. are opportunistic fungi, some species of which are known to have caused severe leaf and shoot blight of many eucalypt species in the tropics and subtropics (Park *et al.*, 2000; Crous *et al.*, 1989). These have a wide host range, but have not been recorded in Australia (Sankaran *et al.*, 1995). *Aulographina eucalypti* infects many eucalypt species and causes leaf spotting of mature leaves which can lead to severe defoliation in the subtropics (Crous *et al.*, 1989). This pathogen is present in the eastern states and has been isolated from *E. globulus* in southwestern Australia (Sankaran *et al.*, 1995; Davison, 1995). *Puccinia psidii* (the guava rust fungus) is a relatively new pathogen of eucalypts and has a wide host range (Coutinho *et al.*, 1998; Crous *et al.*, 1989). To date, this rust has not been recorded in Australia (Sankaran *et al.*, 1995). *Mycosphaerella* spp. are associated with eucalypt leaf spots world-wide (Crous *et al.*, 1998; Park and Keane, 1982), including Australia (Park, 1984). These fungi cause small irregular leaf spots, to large spreading lesions, premature defoliation and twig cankers (Davison, 1995). When infection is severe, these pathogens cause defoliation resulting in reduced tree growth (Maxwell *et al.*, 2000; Davison, 1995). Mycosphaerella leaf disease (MLD) is geographically widespread within *E. globulus* plantations in WA (Maxwell, 2003). The incidence of MLD has increased significantly since it was first reported in WA and is now an increasing problem for plantation managers, causing considerable damage to juvenile foliage in some areas (Maxwell *et al.*, 2000). As the climatic conditions of WA are similar to those in some areas of South Africa, where this disease has caused significant damage, it should be considered a serious threat to *E. globulus* in WA plantations.

Stem diseases

World-wide, the major fungi which cause serious stem diseases in plantation-grown eucalypts are *Cryphonectria cubensis* (Bruner) Hodges and *Erythricium (Corticium)*

salmonicolor (Berk. and Broome) Burds. (Old and Davison, 2000). In Australia, *Er. salmonicolor* has not been recorded on eucalypts, however it is common on fruit trees in New South Wales (Old and Davison, 2000) and *C. cubensis* has been recovered from root cankers and a crown canker of *E. marginata* (jarrah) in southwestern Australia (Davison and Coates, 1991).

Cryphonectria canker, caused by *C. cubensis,* is the most important stem canker pathogen of plantation eucalypts in the tropics and subtropics (Wingfield *et al.,* 2001; Old and Davison, 2000; Wingfield *et al.*, 1989; Boerboom and Maas, 1970). It has been recorded on many eucalypt species from Surinam, Brazil, Venezuela, Cuba, Puerto Rico, Florida and Hawaii, Trinidad and Western Samoa, Hong Kong and Thailand, Sumatra and India and the Cameroon (Old and Davison, 2000). Records from South Africa (Wingfield *et al.*, 1989; Conradie *et al.*, 1990) and southwestern Australia (Davison and Coates, 1991) are the only ones of this pathogen in more temperate climates. *Corymbia maculata* (Spotted gum) and *E. saligna* (Sydney blue gum) are highly susceptible and *E. grandis* (Flooded gum) is moderately susceptible to this pathogen (Hodges *et al.*, 1979). *Cryphonectria cubensis* has severely limited the development of plantations using susceptible *Eucalyptus* spp. in areas where climatic conditions favour the disease (Wingfield *et al.*, 2001; Conradie *et al*., 1990). The main economic effects of disease caused by *C. cubensis* in plantations are reduced growth rate, reduced coppicing, increased tree mortality, all of which ultimately result in reduced wood yield (Old and Davison, 2000). In Australia, *C. cubensis* has been recovered from root cankers and a crown canker of *E. marginata* in native forest in WA (Old and Davison, 2000; Davison and Coates, 1991), but has not been recovered from *E. globulus*.

Pink disease, caused by *Er. salmonicolor,* is a severe disease of woody plants, including eucalypts, in the tropics and sub-tropics (Old *et al.*, 2003; Davison, 1995; Morgan, 1994). This pathogen is present in India, Africa, New Zealand, Brazil and Australia, however it has not been recorded in WA (Shivas, 1989). Pink disease has been reported in two- to five-year-old *E. globulus* plantations in North Bengal, where it caused over 50 per cent mortality (Morgan, 1994). It is considered that if *Er. salmonicolor* was introduced into southwestern Australia, it would not present a severe risk to *E. globulus* plantations, as the environmental conditions of the region are not conducive to the survival of the pathogen (Davison, 1995).

The most commonly isolated fungi from stem and branch cankers (necrotic bark and shoot material showing dieback symptoms) of several eucalypt species in WA are *Endothiella eucalypti*, the anamorph of *Cryphonectria eucalypti* Gryzenhout (previously *Endothia gyrosa*), *Botryosphaeria ribis* Grossenb. and Dugg. and *Cytospora eucalypticola* Van der Westhuizen (Davison and Tay, 1983; Old *et al.*, 1990; Davison, 1995). Davison (1995) reported that these fungi were present on healthy, vigorously growing trees, but have the capacity to cause severe disease in trees subject to environmental stress.

Endothiella eucalypti is capable of causing severe disease on eucalypt stems and branches in WA (Old and Davison, 2000). *Endothiella eucalypti* is the anamorph of the newly classified *C. eucalypti* which is found in mainland Australia, Tasmania and South Africa and occurs exclusively on eucalypt species (Gryzenhout *et al*., 2003). The pathogen was previously incorrectly known as *Endothia gyrosa*, an opportunistic canker pathogen of various hardwood species in the USA (Venter *et al.*, 2001; Roane *et al.*, 1974). Recent phylogenetic studies have shown that the fungus identified as *End. gyrosa* on eucalypts from Australia and South Africa was not the same species as that from North America (Venter *et al.*, 2001). This led to the description of the new species *C. eucalypti* (Gryzenhout *et al*., 2003) which is widely associated with stem and branch cankers on eucalypts in south-eastern Australia. However, to date only its anamorph, *En. eucalypti,* has been found in WA (Yuan and Mohammed, 2000; Davison and Coates, 1991).

Cryphonectria eucalypti was originally described as *Endothia havanensis* on *E. marginata* and *E. saligna* (Davison, 1982), but was later identified, by isozyme analysis, as the *End. gyrosa* anamorph, *Endothiella gyrosa* (Davison and Coates, 1991). Previously, differentiating between species of *Endothia* and *Cryphonectria,* two closely related genera, which share the *Endothiella* anamorph, has lead to confusion, especially in the absence of the teleomorph (Venter *et al*., 2001; Walker *et al*., 1985; Davison, 1982). In 1978, Barr separated *Endothia* and *Cryphonectria* on the basis of differences in ascospore and stromatal morphology. There has been much confusion in the literature concerning the naming of this fungus. However, through the use of molecular and morphological characteristics, *End. gyrosa* present in Australia and South Africa has been classified as a *Cryphonectria* species and has been named *C. eucalypti* (Venter *et al.*, 2002; Venter *et al.*, 2001).

Cryphonectria eucalypti has a wide host range among eucalypt species and has been found in *E. grandis, E. nitens* (Shining gum) and *E. urophylla* as well as hybrids of *E. grandis* with *E. camaldulensis* (River red gum) (Van der Westhuizen *et al*., 1993). *Cryphonectria eucalypti* is considered native to Australia (Davison and Tay, 1983), however, there is controversy surrounding its origin (Walker, 1987). In WA, only the *C. eucalypti* anamorph (*Endothiella*) has been found. This thesis will refer to it as *En. eucalypti*.

Botryosphaeria ribis is often recovered from cankers on eucalypts (Yuan and Mohammed, 1997). It is known to have a wide host range and is widespread in temperate and tropical regions of the world, including Australia (Old and Davison, 2000; Morgan, 1994; Shearer, 1994). *Botryosphaeria ribis* is thought to be an introduced pathogen to southwestern Australia (Shearer, 1994; Davison and Tay, 1983). It causes twig, branch and trunk cankers, which may develop to girdle branches and stems (Fraser and Davison, 1985; Davison and Tay, 1983). Pathogenicity studies on eucalypts have indicated that *B. ribis* is an opportunistic pathogen. However, *B. ribis* was associated with the death of *E. radiata* (Narrow -leaved peppermint - native to eastern Australia) grown in species selection trials in WA (Shearer *et al.*, 1987). In addition, a *Botryosphaeria* sp. has been associated with cankers on *E. globulus* in plantations in Portugal and *B. ribis* was identified as the causal agent of canker and crown dieback in young (18 months old) *E. globulus* in WA (Morgan, 1994). Environmental stresses, such as drought, have been associated with predisposition of the host to infection with *Botryosphaeria* spp. (Ma *et al.*, 2001; Smith *et al.*, 1996b; Smith *et al.,* 1994; Crist and Schoeneweiss, 1975).

Cytospora eucalypticola, a Coelomycete, is one of the most commonly recovered fungi from eucalypts in both eastern and western Australia and has a wide host range (Yuan and Mohammed, 1997; Morgan, 1994; Shearer, *et al.*, 1987; Old *et al.*, 1986; Fraser and Davison, 1985; Davison and Tay, 1983). This fungus also occurs in Argentina, Brazil, India, New Zealand, Solomon Islands, South Africa and Zambia (Old and Davison, 2000). Pathogenicity studies on seedlings and trees indicate this fungus to be a non-aggressive facultative parasite (Morgan, 1994; Old *et al*., 1990; Fraser and Davison, 1985; Davison and Tay, 1983). *Cytospora* spp. are able to survive for long periods of time in wounds and endophytically in healthy tissue (Guyon *et al.*, 1996; Shearer, 1994; Bettucci and Saravay, 1993; Fraser and Davison, 1985; Davison and Tay, 1983). Damage caused by this fungus is often closely associated with a stressed host (Roux *et*

al., 2000; Yuan and Mohammed, 1997; Guyon *et al.*, 1996; McIntyre *et al.*, 1996). Determining the initial cause of stem cankers is sometimes difficult, as the fungi isolated from cankered tissue, such as *C. eucalypticola,* may be secondary invaders of the already diseased tissue (Davison and Tay, 1983).

What is a canker?

Cankers were defined by the Federation of British Pathologists in 1973 as 'sunken necrotic lesion(s) of main roots, stems or branches arising from disintegration of tissue outside the xylem cylinder, but sometimes limited in extent by host reactions which can result in more or less massive outgrowth of surrounding tissues' (Old and Davison, 2000). Once the phloem and sapwood have been invaded, cankers appear as sunken, cracked tissue with the associated phloem discoloured dark brown. Fungi which cause cankers are primarily associated with the bark, or bark and cambium (Old and Davison, 2000; Shearer, 1992). Kino, an exudate rich in a range of polyphenolics, may be associated with cankers and is the characteristic response to cambial damage in eucalypts (Old and Davison, 2000). The Proteaceae and Myrtaceae families are particularly susceptible to canker fungi. The fungi sporulate on dead bark and the sexually-produced ascospores are dispersed by the wind and the asexually-produced pycnidiospores in rain splash (Shearer, 1994). It is thought that canker-causing fungi require an opening for entry such as a wound or lenticel in order to cause disease (Yuan and Mohammed, 2001; Shearer, 1994).

Canker types can be divided into (i) annual, (ii) perennial or (iii) diffuse (Old and Davison, 2000; Shearer, 1994; Fraser and Davison, 1985). An annual canker is contained by the host defences within the first year of invasion (Old and Davison, 2000; Shearer, 1994). If the plant is stressed and unable to launch a full defence response to the pathogen, a perennial canker is the result. Perennial cankers can be identified by the concentric rings formed as the host defends itself from successive pathogen attacks. The pathogen is able to survive on dead tissue until it has the opportunity to re-invade the healthy tissue in later years. Diffuse cankers are the result of rapidly invading fungi which provoke a minimal or ineffective host response. These cankers cause extensive lesions which may girdle stems and lateral branches (Old and Davison, 2000).

Stresses which may predispose eucalypts in plantations to disease

The complex interactions between the environment, the host and the pathogen are illustrated in the disease triangle (Brown and Ogle, 1997). If the environmental

conditions reduce the resistance of the host to the pathogen, the balance favours the pathogen and disease is the result. There are numerous environmental factors which affect the growth of *E. globulus*, such as light intensity, carbon dioxide supply, soil type, nutrient and water supply, salinity and waterlogging. Most eucalypts are well adapted to recovering from environmental stresses such as periodic burns, drought and attack by insects. However, in a plantation environment with fast growing trees of uniform age, growing outside their normal range, the impact of these environmental stresses is magnified (Shearer, 1994). The high incidence and severity of cankers in some eucalypt plantations has been directly attributed to environmental stress (Old and Davison, 2000). There are three main environmental factors which have a significant impact on the health of plantations within southwestern Australia: (i) water deficit (drought); (ii) nutrient imbalance (particularly copper deficiency); and (iii) defoliation (by insects or foliar disease). The severity of each of these environmental stresses varies according to the genetics of the host, however all these factors reduce the vigor of *E. globulus* and render it more susceptible to canker-causing fungi.

(i) Water Stress

Eucalyptus globulus are well adapted to short periods of water stress, punctuated by rainfall, but are vulnerable to prolonged dry periods (White *et al.*, 1999). This presents a challenge in managing *E. globulus* plantations in southwestern Australia, as the hot dry summers of this region are in stark contrast to the temperate climate with uniform rainfall distribution this species experiences in its natural environment. Rainfall in southwestern Australia is variable and long-term averages do not necessarily indicate water availability to plantations (Crombie and McGrath, 1999). Drought periods or drought years occur frequently (McGrath, 1999). Water availability, rainfall and evaporative demand, combined with the water storage capacity of soils (which is determined by soil type, depth and topography) are the main factors which influence the growth and survival of *E. globulus* in plantations (Crombie and McGrath, 1999; White *et al*., 1999). Therefore, water limitation, rather than excess water (waterlogging) is considered a serious limiting factor for *E. globulus* growth. To date, no cases of canker disease associated with waterlogging have been documented.

Plantations which have a substantial amount of water available at the end of the summer months in a normal year will be less likely to experience severe water deficits in dry years (Crombie and McGrath, 1999). Root depth also influences the survival of *E. globulus* under drought conditions. However, while the site conditions leading to

drought stress have been identified, deaths due to drought are still occurring in *E. globulus* plantations in southwestern Australia (White *et al*., 1999). Therefore, careful site selection and predictions of site productivity are vital to maximise plantation productivity.

Previous studies have shown that drought-stressed trees are more susceptible to canker disease than non-stressed trees (Ma *et al.,* 2001; Schoeneweiss, 1981; Crist and Schoeneweiss, 1975). Water stress is known to affect cell growth, cell division, cell wall synthesis and plant hormone balance and these changes may affect the resistance of trees to disease (Ma *et al.*, 2001). The increased susceptibility of water stressed trees is attributed to the reduced ability of the host to produce a boundary zone in the phloem, which enables the invading fungi to penetrate further into the xylem (Old and Davison, 2000). Drought stress was found to increase the duration that wounds are susceptible to infection. For example, studies on aspen showed that *Cytospora* caused fewer cankers on non-drought stressed trees, with the stressed trees being less able to confine the lesions than the non-stressed trees (Guyon *et al.*, 1996; McIntyre *et al*., 1996).

In order for water stress to increase the susceptibility of the host, the degree of water stress must be severe, with stems having water potentials below the threshold levels (Schoeneweiss, 1981). Water stress predisposition in stems of woody plants was found to be reversible and the host tissues regained resistance to pathogen attack three to five days after the water stress had been relieved (Schoeneweiss, 1981). The duration of water stress (where plant water potentials remained at or below the threshold level) required for increased susceptibility of woody stems was more than three days (Schoeneweiss, 1981). Therefore, in the absence of a pathogen, the host is able to recover from a short period of severe water stress. However, if the pathogen is continuously present within the plantation environment, the stressed host is highly vulnerable, even to non-aggressive pathogens.

Eucalypts exhibit a different pattern of resistance under drought stress than the aspen mentioned above. For example, *C. cubensis* caused smaller lesions on potted drought-stressed *E. globulus* than on non-droughted seedlings (Swart *et al.*, 1992). It has been reported that some *E. grandis* clones were less susceptible to *C. cubensis* due to their ability to withstand drought (Swart *et al*., 1992). Glasshouse studies in WA on *E. globulus* showed that droughted plants were less susceptible to infection with an *Endothiella* sp. than plants held at field capacity (Lawrence, 1998). Water relations

appear to play an important role in determining the ability of the host to respond to pathogen challenge. The mechanisms behind this stress-induced susceptibility and variation in response of different hosts to drought stress, remain unclear.

(ii) Nutrient stress

With the demand for expansion of *E. globulus* plantings in southwestern Australia, some plantations are being established on sites with nutrient poor soils. With the addition of fertilizers with macronutrients such as N and P in forms low in trace elements, secondary micronutrient deficiencies develop (Dell, 1994; Turnbull *et al.*, 1994). Although limited studies have been conducted on nutrient deficiencies of eucalypts, disorders due to nutrient imbalances (N or Cu) in eucalypt plantations have been reported previously (Gherardi *et al*., 1999; Shedley *et al*., 1995; Dell, 1994; Turnbull *et al.*, 1994).

Copper deficiency, due to low or poorly available soil copper, is the main micronutrient deficiency which has been identified in *E. globulus* plantations in southwestern Australia and is often associated with ex-pasture sites (Rogers, 2002; Gherardi *et al*., 1999). It has also been identified as the cause of distorted growth of two-three-year-old stands of *Corymbia maculata* in bauxite rehabilitation sites in WA (Dell, 1994). Copper deficiency is common on acidic sandy duplex soils that are low in organic carbon. It also occurs on soils with a surface peaty horizon that are subject to waterlogging in winter, where copper is largely unavailable after the formation of humic or calcareous complexes (Gherardi *et al.*, 1999). Deficiency symptoms include stem and branch deformities, marginal necrosis in young fully expanded leaves (YFEL), deformed leaf margins, twisting of stems and branches and death of lateral shoots (Dell, 1994). The deformities resulting from copper deficiency reduce the amount of merchantable wood, thereby reducing plantation productivity.

Copper is an essential micronutrient for the development of plants. It plays a key role in photosystem 1 of the photosynthetic pathway and in lignin biosynthesis, and therefore it is likely to have a role in host defence (Gherardi *et al.*, 1999). Studies have linked copper deficiency in woody plants to a reduction in lignification (Dell, 1994; Downes and Turvey, 1986). Copper deficiency has also been associated with reduced disease resistance of plantation *E. globulus* (Dell *pers. comm.*). Studies undertaken in the glasshouse have indicated an increased susceptibility of copper-deficient *E. globulus* to canker-causing fungi (Ishaq, 1999). Therefore, the combination of the

copper deficiency-induced stem and branch deformities and increased susceptibility of copper-deficient plantation grown *E. globulus* to disease indicate that copper deficiency has the potential to have a major impact on plantation productivity.

(iii) Defoliation Stress

Carbohydrate derived from photosynthesis can be allocated to growth and metabolism or to the accumulation of reserves. The balance between these processes is easily disturbed (Turner *et al*., 2001). A reduction in foliage surface area and photosynthetic capacity have been shown to reduce the general health and growth rate of the host tree, altering water relations and influencing allocation of photosynthate (Chen *et al.*, 2001; Guyon *et al.*, 1996). For example, defoliation of *E. regans* and *E. grandis* saplings reduced the concentration of soluble carbohydrates and starch, which in turn, reduced the ability of the host to produce effective wound barriers (Old *et al*., 1990). The longer the period of defoliation stress, the more susceptible the host becomes as reserves are expended to maintain basic growth requirements (Old *et al.*, 1990; Crist and Schoeneweiss, 1975). Defoliation of *E. globulus* in a plantation environment is primarily due to insects or foliar pathogens (such as *Mycosphaerella* spp.). Plantation eucalypts, grown on sites out of their natural range, may be exposed to a new suite of insect pests and diseases.

The juvenile foliage of *E. globulus* is particularly sensitive to insect attack. More than 85 insect species have been recorded as pests in eucalypt plantations (Strauss, 2001). Leaf damage is predominantly caused by insect chewers and skeletonisers such as Autumn Gum Moth (*Mnesamplea privata*), Leafblister Saw Fly (*Phylacteophaga froggatti*), Weevils (Genera: *Gonipterus* and *Oxyops*) and the Chrysomelid beetles (Genera: *Chryophtharta* and *Paropsis*) (Maxwell *et al.*, 1998; Farrow *et al.*, 1994). The timing of the defoliation event can be very important (McIntyre *et al*., 1996; Schoeneweiss, 1981). For example, Autumn Gum Moth attacks juvenile foliage during autumn, which can not be replaced until spring. Damage to *E. globulus* decreases significantly as the trees enter their adult foliage stage, as the larvae do not feed on adult foliage. However, long periods without adequate juvenile foliage can have a negative effect on the growth and development of the tree (Farrow *et al.*, 1994). As the length of time in plantation and the number of hectares planted to *E. globulus* increases, it is likely that additional pests will also increase (Strauss, 2001). Therefore, early identification and detection of potentially damaging insect populations is essential for management of plantations of young *E. globulus* (Wills and Burbidge, 1998).

As mentioned previously, *Mycosphaerella* species are the main leaf pathogens responsible for significant defoliation in eucalypt plantations. *Mycosphaerella* spp. form necrotic lesions on the leaves and young shoots of eucalypts, and these can lead to serious defoliation and stunt tree growth (Maxwell *et al.*, 2000; Davison, 1995). Mycosphaerella leaf disease has the potential to reduce the photosynthetic capacity of trees to such an extent that death eventually occurs. *Eucalyptus globulus* is particularly susceptible to this pathogen (although no deaths have been reported in WA), especially the juvenile foliage which has been severely affected by MLD (specifically caused by*M. nubilosa*) in WA plantations (Maxwell *et al.*, 2000). Plantations of *E. globulus* in Tasmania have also experienced severe defoliation of juvenile leaves due to MLD (specifically caused by *M. cryptica* and *M. nubilosa*) (Milgate *et al.*, 2001). However, adult leaves are also susceptible to this pathogen, with *M. cryptica* having a serious impact on the health of adult leaves in *E. globulus* plantations in WA (Maxwell *et al.*, 2000). Therefore, management of these pathogens, and thus the degree of defoliation imposed on *E. globulus,* will aid in sustaining plantation growth and resistance to non-aggressive pathogens.

Loss of foliage predisposes plants to attack by non-aggressive pathogens (Old and Davison, 2000; Schoeneweiss, 1981; Crist and Schoeneweiss, 1975). Studies by Old *et al.* (1990) have shown that canker-causing fungi such as *En. eucalypti* and *B. ribis* induced more severe disease on defoliated *E. regnans* (Mountain ash) and *E. delegatensis* (Alpine ash) seedlings and saplings than non-defoliated trees (Old *et al.*, 1990). Defoliation stress has been associated with increased canker expansion in other plant species such as *Populus tremuloides* (Quaking aspen) inoculated with *Cytospora chrysosperma* (McIntyre *et al.*, 1996) and *Betula alba* (European white birch) inoculated with *B. ribis* (Crist and Schoeneweiss, 1975). In contrast, results from a preliminary glasshouse defoliation trial conducted on *E. globulus* seedlings suggest that complete (100%) defoliation, as a single event, enhanced the resistance of the host to infection with an *Endothiella* sp. (Lawrence, 1998). Seedlings with one third of their leaves removed had significantly larger lesions and a higher level of girdling than more heavily defoliated trees. This result was supported by a later study on *Corymbia calophylla* (marri) seedlings, which showed that complete defoliation limited the extent of colonisation of the stem by *Endothiella* sp. compared to non-defoliated seedlings (Paap, 2001). In conclusion, the impact of foliage loss through insects or leaf pathogens appears to vary according to the host. However, the mechanisms involved in

host response to defoliation and subsequent susceptibility to non-aggressive pathogens remain unclear.

Thesis rationale

The complex interactions between disease, stress and host resistance influence the success of *E. globulus* as a plantation species in southwestern Australia. Although the *E. globulus* industry is still young in WA, the levels of disease within the plantation estate are increasing (Maxwell *et al.*, 1998). As the area required for *E. globulus* planting increases, plantations are being established in more marginal areas outside of the species preferred range. This exposes *E. globulus* to environmental conditions to which it is not suited, such as prolonged periods without adequate rainfall and a new suite of insects and diseases. Environmental stress has been highlighted as a key factor in the successful disease development of weakly pathogenic canker-causing fungi. The even-aged monoculture, typical of the plantation environment, leaves it prone to endemic disease outbreaks (Heather and Griffin, 1984). Therefore, establishing the extent to which *E. globulus* is able to adapt and recover from environmental stresses, without affecting its resistance to opportunistic pathogens, is vital for future provenance and plantation site selection. Although the impact of stem diseases within *E. globulus* plantations throughout southwestern Australia is currently at relatively low levels, our knowledge of the nature of disease accumulation over time and evidence from around the world indicate that as the plantation industry ages, the potential for disease epidemics increases markedly. The very nature of *E. globulus* as a plantation species, in that it may be coppiced, exposes this species to increasing disease buildup in the soil, decaying leaf or excised stem material.

Previous surveys of eucalypts in southeastern Australia (Shearer, 1994; Davison and Coates, 1991; Fraser and Davison, 1985; Davison and Tay, 1983) have indicated that *En. eucalypti, B. ribis* and *Cy. eucalypticola* are commonly isolated canker-causing fungi affecting native forests and plantations. However, detailed studies of the canker-causing fungi present in *E. globulus* plantations and the environmental factors associated with their survival, spread and ability to cause serious disease have not been undertaken. Therefore, the initial aim of the current study was to survey *E. globulus* plantations across southwestern Australia and to identify the major fungi responsible for causing cankers, and the level of disease present. Once the primary canker-causing pathogen(s) were identified, the impact of host genetics, nutrient stress and defoliation

on the susceptibility of *E. globulus* to disease could then be assessed. More specifically the complete aims of this study were to:

- conduct a survey of *E. globulus* plantations across southwestern Australia and assess damage caused by canker fungi (Chapter 2);
- identify and characterise the main canker-causing fungus using morphological and molecular methods (Chapter 2);
- determine the pathogenicity of the canker-causing fungus to *E. globulus* provenances in the glasshouse and in the field (Chapters 3 and 4);
- examine the role of wounding in the infection process of the canker-causing fungus (Chapter 5);
- investigate the population genetics of the fungus (Chapter 6); and
- investigate the impact of environmental stresses such as copper deficiency and defoliation on the susceptibility of *E. globulus* to the canker-causing fungus (Chapters 7 and 8).

Chapter 2

Fungi associated with canker disease in *Eucalyptus globulus* plantations in southwestern Australia

INTRODUCTION

In Western Australia, the contribution of canker fungi to the death of native and plantation eucalypts has largely been ignored, despite an increase in the dieback of native eucalypts in the region since the 1970's (Shearer, 1994; Kimber, 1980). Although several studies have been conducted in eastern Australia (Old *et al.*, 1986; Yuan and Old, 1995; Yuan and Mohammed, 1997; Yuan and Mohammed, 2000), the number of in-depth surveys investigating the incidence of canker fungi and potential disease threat to plantation *Eucalyptus globulus* in southwestern Australia has been limited.

Davison and Tay (1983) conducted the first major study of twig, branch and upper trunk cankers of *E. marginata* within the Northern Jarrah Forest and Swan Coastal Plain during 1981 and 1982. In their study, canker tissue from trunks and branches of introduced eucalypts, such as *E. globulus* and *E. saligna,* were also collected. *Endothiella eucalypti, Botryosphaeria ribis, Cytospora eucalypticola* and *Discosporium eucalypti* were the main fungi isolated. *Cytospora eucalypticola* was the most commonly isolated fungus, however, only *En. eucalypti* and *B. ribis* were subsequently proven to be pathogenic in coppiced jarrah and saplings in the field (Davison and Tay, 1983). The researchers suggested that these fungi, apart from *B. ribis,* are part of the indigenous fungal flora of eucalypts in the region (Davison and Tay, 1983).

Ten small *E. saligna* plantations on rehabilitated mine sites in the Northern Jarrah Forest were surveyed for stem cankers in 1982 and 1983 by Fraser and Davison (1985). Cankers were recorded on 43% of trees surveyed, with *En. eucalypti, B. ribis* and *Cy. eucalypticola* being the main fungi isolated from both perennial and annual cankers. All three fungi were able to cause cankers in subsequent pathogenicity tests, however as identified previously (Davison and Tay, 1983), *En. eucalypti* and *B. ribis* induced larger lesions more frequently than *Cy. eucalypticola* (Fraser and Davison, 1985).

In 1995, a review of the fungal pathogens which pose a potential threat to *E. globulus* plantations in southwestern Australia was conducted (Davison, 1995). At that time,

En. eucalypti, B. ribis and *Cy. eucalypticola* were present in low levels on healthy trees, but it was concluded that these fungi were only capable of causing disease on trees stressed by adverse environmental conditions (Davison, 1995). Three years later, a general health survey was conducted on one- and two-year-old *E. globulus* over six plantations in the Albany region. This study established that 16% of trees surveyed were exhibiting symptoms of canker infection (Maxwell *et al.*, 1998). Another survey by Tovar (1998) of canker incidence in 14 *E. globulus* plantations in WA isolated *Endothiella* (27% of canker cases), *B. ribis* (10%) and *Cy. eucalypticola* (51%) most commonly from cankered tissue. Pathogenicity tests with these fungi indicated that the isolates of the *Endothiella sp*. were the most likely agent to cause canker disease in *E. globulus* (Tovar, 1998).

More recently, Paap (2001) reported that cankers were severe and widespread in *Corymbia calophylla,* a keystone species in the jarrah forest, in southwestern Australia. Girdling cankers were responsible for the death of trees at five of the six sites surveyed (Paap, 2001). Cankers were present on trunks, main branches and smaller branches, with *Cy. eucalypticola* (55% of cankers) and *Endothiella* (50%) isolated most frequently from diseased tissue (Paap, 2001). Pathogenicity tests showed again that the *Endothiella sp.* was the most aggressive pathogen isolated.

With the recent and ongoing expansion of the *E. globulus* plantation estate in southwestern Australia, assessment of the current impact of canker fungi on plantation health is required. Therefore, the aim of the current study was to identify the major canker-causing fungi and the key environmental factors which contribute to canker incidence and severity within the *E. globulus* plantation estate in southwestern Australia.

MATERIALS AND METHODS

Experimental design

A preliminary survey of two three- to four-year-old *E. globulus* plantations (50 trees at each plantation) within the Albany region (selected according to soil-type and rainfall) was conducted to establish canker incidence, assess general plantation health and identify fungi commonly associated with cankers. Twenty-six, two- or three-year-old *E. globulus* plantations located in six regions of southwestern Australia were then selected. Canker incidence and severity was recorded in two compartments of each plantation, with 50 trees assessed per compartment. Samples of bark and stem tissue

were collected where cankers were present and returned to the laboratory for isolation of causal pathogen(s). Putative fungal pathogens were identified by morphological and DNA sequencing analysis.

Selection of* Eucalyptus globulus *plantations

The six main geographical regions where *E. globulus* plantations occur within southwestern Australia (Albany, Bunbury, Denmark, Esperance, Manjimup and Margaret River) were systematically surveyed for canker-causing fungi. The southwest was divided into 20 km^2 grids and, within each of the geographical regions, a two- or three-year-old *E. globulus* plantation selected from within each 20 km^2 area. Rainfall and evaporation rates at each plantation location, within each grid, were also considered during plantation selection. Six plantations were selected from the Albany region, three from Bunbury, seven from the Denmark region, two from Esperance, five from Manjimup and three from the Margaret River region (Figure 2.1; Table 2.1). The number of plantations per region was dictated by the size of the region and number of plantations able to be selected within the grid system, as some grid sections did not have a plantation of suitable age. Two- and three-year-old plantations were chosen for sampling as previous studies indicated that plantations of this age had a higher incidence of canker disease than older or younger plantations (Maxwell *et al.*, 1998). In addition, as the WA plantation estate is young, plantations of this age were well represented throughout the estate. In the majority of cases, plantations were three years old, however, two-year-old plantations were sampled when older plantations were not available within the grid system. At two and three years of age, *E. globulus* saplings are in transition between juvenile and adult leaf phenology. The sampling was conducted from mid December 1999 to mid April 2000 and site characteristics recorded (Table 2.1).

In addition to those selected from the 20 km^2 grid system, three plantations (one in the Albany region - plantation 23, and two in the Denmark region - plantations 6 and 7), were surveyed, as they belonged to a different plantation company from the remainder of the plantations surveyed within that region. At these plantations, a smaller survey was conducted for comparison of disease incidence and severity, with a total of 50 trees in one transect, at one compartment (Table 2.1).

Table 2.1. Details of plantation site, year of planting, rainfall of area, provenance, soil type and general observations relating to the *Eucalyptus globulus* plantations surveyed throughout southwestern Australia.

Region/ Plantation Number (Fig 2.1)	Year of planting	Rainfall (mm)	Provenance	Soil Type	Observations/History
Albany 19	1997	670	Flinders Island	T1=gravelly sand over lateritic clays to deep anaerobic subsoil T2=gravelly sand over lateritic clays to deep anaerobic subsoil.	Borer damage associated with 3 cankers, some evidence of copper deficiency (stem deformation). History of clover and grass pastures with regular P application.
20	1994-1995	775	Trial material	T1= Redmond lateritic duplex T2=waterlogged sand	Progressively cleared in 1950's and 1960's. Mixture of annuals and perennials sown with regular fertilizer application.
21	1997	749	Flinders Island	T1=valley sand T2=sandy gravel spongelite and Aeolian sand	Progressively cleared in 1960's and 1970's. Regular fertilizer application since.
22	1997	750	Flinders Island	N/A	Gradually cleared since 1960's. Regular application of fertilizer.
23	N/A	755	Unknown	N/A	Progressively cleared in 1960's and 1970's. Regular fertilizer application since.
24	1997	N/A	N/A	N/A	Leaf blister saw fly severe on juvenile leaves
Bunbury 1	1997	N/A	Killiecrankie	N/A	N/A
2	1997	N/A	Killiecrankie	Sand	History of grazing.
3	1997	N/A	Greeveston	N/A	N/A
Denmark 12	1998	950	King Island	T1=deep sandflats and slopes	MLD slight to moderate, no MLD on adult leaves, slight broomsticking (Cu deficiency). Progressively cleared since 1969. Regular fertilizer application since.
13	1997	N/A	Greeveston	Karri loam	>80% defoliation on juvenile leaves predominantly due to MLD, some resprouting of juvenile leaves. Adult foliage <12% MLD in lower canopy, symptoms of nutrient deficiency such as stunting and stem deformation, tip dieback severe. History of grazing

Table 2.1 contd.

Region/ Plantation Number (Fig 2.1)	Year of planting	Rainfall (mm)	Provenance	Soil Type	Observations/History
Denmark contd. 14	1998	N/A	Otways	Duplex soils	Some herbicide damage on edges of tree rows, nutrient deficiency (loss of apical dominance), leaf curl. Minimal MLD (<1.5% foliage affected) History of grazing.
15	1988	900	King Island	T1=duplex soil with shallow laterite slopes	Minimal MLD, no adult MLD, healthy slow growing trees. Progressively cleared since late 1960's. Regular fertilizer application since.
16	1997	N/A	Greeveston	Karri loam	80% defoliation of juvenile leaves predominantly due to MLD, some resprouting of juvenile leaves. History of grazing.
17	1997	N/A	Greeveston	varies from Karri loam to sand over clay	>80% defoliation of juvenile leaves predominantly due to MLD. History of grazing.
18	1998	N/A	Otways	N/A	Minimal MLD, no cankers, dense fern undergrowth. No defoliation due to MLD, tree growth stunted with broad lateral growth
Esperance 25	1997	650	Unknown	T1+T2=longitudinal sand dunes slope 2-6%, relief 3-5m rapidly drained	Trees showing copper deficiency symptoms, moderate MLD damage.
26	1997-1998	650	Flinders Island	T1=well drained sandy duplex (SQ2) T2=deep waterlogged sandy duplex (SQ3)	Moderate MLD damage
Manjimup 7	1997	N/A	Otways	Sandy gravel	History grazing/crop.
8	1997	N/A	W142 (unknown)	Gravel loams	History of grazing.
9	1998	N/A	N/A	N/A	Stunted trees in low lying areas of plantation
10	1997	N/A	N/A	N/A	Poor tree form, nutrient deficient (stem deformation). Many trees shedding blackened (measled) bark.

Table 2.1 contd.

Region/ Plantation Number (Fig 2.1)	Year of planting	Rainfall (mm)	Provenance	Soil Type	Observations/History
Manjimup contd. 11	1997	N/A	Greeveston	Karri loam	Trees small, thin, severe defoliation of juvenile leaves by MLD, some resprouting. Many trees shedding blackened (measled) bark. MLD on adult leaves. History of grazing.
Margaret River 4	1998	N/A	Otways	Sandy loam over clay	History of grazing.
5	1997	N/A	Otways	Sandy loam	History of grazing.
6	1997	N/A	Greeveston	Sand	History of grazing.

Key:
MLD - *Mycosphaerella* leaf disease
T - Transect
N/A - information not available

Survey and transects

Within each plantation, a transect of 50 trees (single row) per compartment was surveyed, with two randomly selected compartments sampled per plantation (with the exception of plantations 6, 7 and 23 where only one transect was undertaken as outlined above). The transects commenced five trees in from the outer edge of the plantation to minimise potential perimeter effects. Each of the 50 trees per transect was assessed according to: (i) form (single or multiple stem); (ii) presence/absence of cankers on the trunk or branches; (iii) type of canker (if present) - measle or basal (Figure 2.2); and (iv) canker severity. Measle cankers were defined as shallow brown/reddish blemishes which occurred on the main trunk or branches and were not generally associated with fruiting bodies (Figure 2.2A). Basal cankers were located at the base of the trunk (Figure 2.2B and C) and fruiting bodies were often visible. Canker severity was rated as (i) slight, (ii) moderate or (iii) extreme (Table 2.2). General observations were made on the overall health of the stand at all sites. These included symptoms of nutritient deficiency (Dell *et al.,* 2001) and insect attack, as well as other disease symptoms such leaf disease cause by *Mycosphaerella* (Table 2.1).

Where a canker was present, a small (≤ 5 cm^2) sample of the cankered tissue (bark and wood) was removed from the trunk or branch with a sterile blade, placed in a sealable plastic bag, returned to the laboratory and processed (within five to seven days).

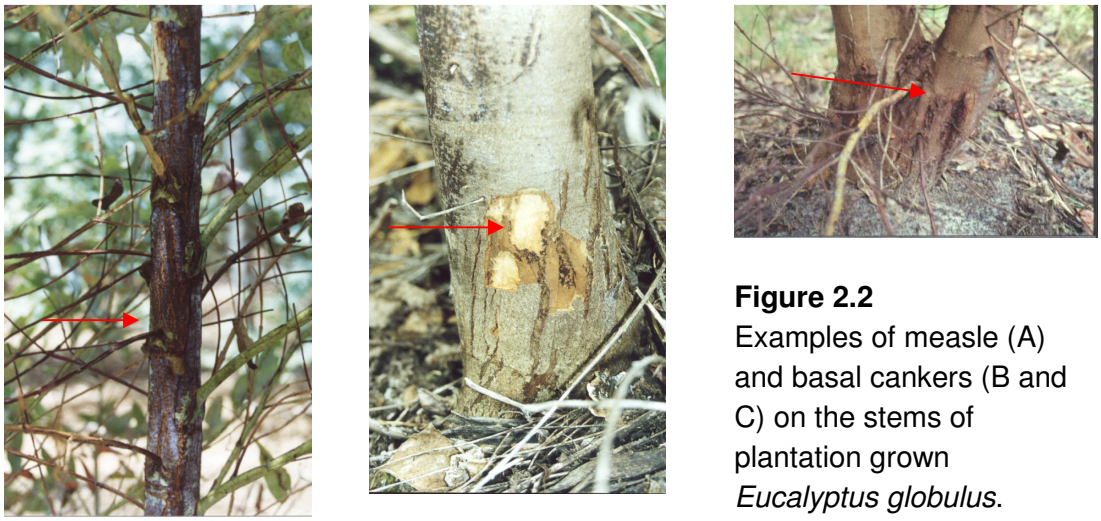


Figure 2.2
Examples of measle (A) and basal cankers (B and C) on the stems of plantation grown *Eucalyptus globulus*.

Table 2.2 Classification of canker severity on branches and stems of plantation *Eucalyptus globulus*.

Canker severity	Lesion diameter (mm)	Depth	Kino* present
1. slight	>40	lesion confined to bark or < 5 mm into wood.	no kino
2. moderate	40-50	lesion extends into wood 5-10 mm.	occasionally
3. severe	≥50	lesion extends well into wood >10 mm, girdling trunk, can result in tree death.	bark cracking, kino present

*The formation of kino, an exudate rich in a range of polyphenolics, is the characteristic response of eucalypts to cambial damage (Old and Davison, 2000; Conradie *et al.*, 1990).

Isolation of causative organisms

In the laboratory, two methods were used to isolate possible causal fungi from cankered bark and wood tissue. Where fruiting bodies were present on the margins of the cankered tissue, canker segments were surface sterilised briefly in 70% ethanol, the fruiting bodies removed using a sterile needle and placed onto half-strength potato dextrose agar (1/2PDA; included 19.5 g/ L PDA and 10 g/ L Agar Grade A; Becton/Dickinson and Co. USA) containing 133 mg/L streptomycin sulphate (Sigma Chemical Co. St Louis, USA). Where fruiting bodies were not visible, canker samples were surface sterilised for a few seconds in 70% ethanol and briefly flamed (1-2 sec) to remove ethanol. Cankered bark and wood segments were then cut into approximately 10 mm^2 segments. Half the segments were placed onto 1/2PDA plus streptomycin (1/2PDA+S) and the other half placed in Petri dishes containing moist sterile filter paper (moist chambers).

Plates containing bark/wood pieces or fruiting bodies and moist chambers were incubated for seven days and fourteen days, respectively, at 24 ˚C under continuous near blue light (NEC T10 Blacklight). If fruiting bodies developed on the bark and wood tissue within the moist chambers, they were removed with a sterile needle, as described above, and placed onto 1/2PDA+S. The fungal mycelium growing from fruiting bodies or stem pieces onto the 1/2PDA+S plates were subcultured onto 1/2PDA and incubated as above.

The fruiting bodies of the most commonly isolated fungi collected in the preliminary survey in the Albany region were examined microscopically and identified to genus using the following literature: Barnett and Hunter (1998); Hanlin (1990); and Sutton (1980). After preliminary sampling the most frequently isolated fungi were *Endothiella* and *Cytospora*.

During the preliminary survey, stem and branch tissue was collected from all trees with canker damage (regardless of the signs of *Endothiella,* such as the presence of orange pycnidia on the bark surface). However, *Endothiella* was chosen as the focus pathogen for subsequent surveys as it (i) was consistently the most commonly isolated pathogen associated with basal cankers of *E. globulus* and (ii) had previously been shown to be more pathogenic than the other frequently isolated fungus, *Cytospora* (Paap, 2001; Tovar, 1998; Fraser and Davison, 1985).

Preparation of single spore isolates

Single spore isolates were used for further morphological and molecular identification of *Endothiella*. The single spore isolates of *Endothiella* were obtained from pycnidia according to the method of Paap, (2001). Briefly, sterile water (5 µL) was placed onto pycnidia of sporulating *Endothiella* cultures and a wire loop was used to agitate the pycnidia to release spores, forming a spore suspension. The spore suspension was then plated onto water agar (15 g/ L) using the streaking method. After two to four days, the germinating spores were observed under a dissecting microscope. Individual germinating spores were removed using a sterile needle, transferred onto 1/2 PDA plates and incubated at 24 °C under continuous near blue light.

***Morphological identification of* Endothiella**

Slides of isolates with different macromorphological characters were prepared and pycnidia were examined microscopically to determine conidia dimensions. Isolates of *Cryphonectria cubensis* supplied by the Department of Conservation and Land

Management (DCLM) were examined as a comparison with the fungal isolates collected in the current study.

Macromorphology

A total of 30 single spore *Endothiella* isolates was randomly selected from the *Endothiella* population collected throughout the WA plantation estate. Six *Endothiella* isolates were randomly selected from Albany, Margaret River and Manjimup, five from Bunbury and Denmark, and two isolates from Esperance (Table 2.3). Two *C. cubensis* isolates were included for comparison (Table 2.3). Three replicate plates were used for each isolate. A 5 mm^2 agar disc from the margin of an actively growing colony of each of the 32 isolates was placed in the centre of a 9 cm diameter 1/2PDA plate and incubated at 24 °C under continuous near blue light for one month. After this time, isolates were assessed for colony morphology, such as the colour and morphology of mycelium and pycnidia distribution.

***Molecular identification of* Endothiella**

DNA extraction

Four *Endothiella* isolates from WA were randomly selected for DNA sequence analysis (E81 - Manjimup, F17 - Esperance, H18 - Bunbury and G2 - Denmark). Four 5 mm^2 agar pieces from an actively growing colony of each of the four isolates were placed into a 1.5 mL microfuge tube and the mycelia ground with a sterile glass rod. The microfuge tube was immersed in liquid nitrogen and the grinding process repeated. DNA was then extracted using a silica binding kit (Ultrapure, Geneworks, Adelaide Australia) according to the manufacturers instructions. A 200 µL aliquot of extraction buffer (200 mmol Tris HCl pH 8.5, 250 mmol NaCl, 25 mmol ethylenediamine tetraacetic acid (EDTA) and 0.5% sodium dodecyl sulfate (SDS); Raeder and Broda (1985)) was added to the microfuge tube and mixed with the homogenate by gentle inversion. The solution was incubated for 1– 1.5 h at 65 ^{o}C and centrifuged at 14 000 *g* (Beckman Microfuge E) for 10 min. The supernatant was transferred into a new microfuge tube containing 600 µL NaI solution and 5 µL of glass milk, briefly vortexed and incubated at room temperature for 10 min with occasional inversion (to precipitate the DNA onto the silica matrix of the glass milk under conditions of high salt and low temperature). The solution was centrifuged for 10 sec at 14 000 *g* and the supernatant discarded. The pellet was washed twice with 600 µL and 200 µL of Bresawash solution (50% ethanol, EDTA, Tris buffer; Bresawash), respectively. Each wash involved briefly vortexing the solution, centrifuging at 14 000 *g* for 10 sec and removing the

supernatant. The pellet was aspirated and resuspended in 20 µL of PCR grade water (UltraPure Water, Fisher biotec, Australia) and incubated at room temperature for 10 min. The solution was centrifuged for 1 min at 14 000 *g* (to precipitate the glass milk) and the supernatant containing the genomic DNA transferred into a sterile 0.5 mL microfuge tube, to which 20 µL of 1ng/ mL RNase (Boehringer Mannheim) was added and the solution incubated at room temperature overnight to digest any RNA present.

The DNA concentration was determined using a Hoefer DyNA Quant 200 fluorometer according to the manufacturers instructions. To confirm the DNA concentration, the samples were electrophoresed on a 1% agarose gel (in TAE buffer) (PROGEN Industries Ltd., Queensland, Australia) at 90 V for 40 min. The size of the DNA bands was determined in relation to a λDNA marker (restricted with *Hin*diii & *Eco.*Ri; Fisher Biotec) as a molecular weight standard. Visualisation of DNA fragments was performed under UV light following gel staining with ethidium bromide (0.5 µg/ mL) for 20 min and destaining in 1x TAE buffer for 10 min. The DNA was then stored at –20 °C until required for Polymerase Chain Reactions (PCR) amplifications.

PCR amplification

The internal transcribed spacer (ITS) region of the rDNA was amplified using the primers ITS1 and ITS 4 (White *et al.,* 1990). Amplification solutions (50 µL) were made aseptically in sterile 200 µL microfuge tubes, containing; 5 ng genomic DNA, 0.2 µM primer, 2.5 mM $MgCl_2$ (Biotech International), 2.5 U *Tth* plus polymerase (Biotech International), 1x polymerisation buffer (Biotech International) equivalent to 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg mL^{-1} gelatin, 0.2 mM dNTPs and sterile, deionised water (UltraPure Water, Fisher biotec, Australia) to make up the reaction volume to 50 µL. These solutions were vortexed for 1-2 sec, then centrifuged at 14 000 *g* for 5 sec. The PCRs were performed in an Applied Biosystems GeneAmp 9600 thermocycler according to the following parameters: initial denaturing step of 96 °C for 2 min; then 30 cycles of 94 °C (30 sec) denaturing, 55 °C (30 sec) annealing, 72 °C (2 min) extension; followed by a 7 min extension cycle at 72 °C, and a hold cycle of 10 °C. Products of the PCR reaction were stored at 4 °C prior to cleaning and sequencing.

To confirm the presence and size of the PCR products, they were electrophoresed on a 1% agarose gel (in TAE buffer) at 90 V for 40 min, as outlined above. Where DNA bands were present, the remaining PCR product was purified using the glass milk

method as outlined for DNA extraction described above, with the following modifications: the PCR product was not incubated in extraction buffer; the volumes of NaI salt, wash, and ethanol solutions were reduced from 600 to 200 µL; and the RNAse digestion step was omitted. The concentration of DNA in the clean PCR product was determined by comparing band intensity against a known amount of marker DNA, visualised on a 1% agarose gel stained with ethidium bromide as described previously.

DNA sequencing

Double stranded ITS fragments were sequenced from each end using an ABI PRISM™ Rhodamine Dye Terminator Ready Reaction Kit in 10 µL sequence reactions according to the manufacturers instructions. Between 80 and 160 ng of purified PCR product and 1.6 pmol of primer ITS 1 or ITS 4 (White *et al.,* 1990) was added to each reaction. Sequencing reactions were performed in an Applied Biosystems GeneAmp 9600 thermocycler according to the following parameters: initial denaturing step of 96 °C for 2 min; then 25 cycles of 94 °C (30 sec) denaturing, 50 °C (5 sec) annealing, 60 °C (4 min) extension; and a hold cycle of 10 °C until collected and precipitated. The products of the sequence reaction were ethanol precipitated. Briefly, sterile 0.5 mL microfuge tubes were prepared with 24 µL of 100% ethanol and 1 µL of Sodium Acetate (10%; pH 5.2). The sequence product was added, vortexed for 5 sec and placed on ice for 20 min to precipitate the DNA. The tubes were centrifuged at 14 000 *g* for 30 min in order to pellet the DNA and the supernatant discarded. The DNA pellet was washed in 250 µL of 70% ethanol, centrifuged at 14 000 *g* for 5 min and the supernatant discarded. The tubes were blotted dry, and dried under vacuum in a rotor speedvac for 10 min. Sequence products were electrophoresed on 5% acrylamide gels. Gels were washed for 10 min with 1000 ml of 20% ethanol, stained and exposed to Kodak SB film. The DNA sequence data was read by an electronic digitizer and aligned by LaserGene version 1.60dz using the CLUSTAR V method. The sequencing of the DNA template was conducted using the software package, Sequencase v 2.0.

Phylogenetic analysis

The forward and reverse sequence data for each isolate were edited and aligned with SEQUED V1.04 (PE Applied Biosystems, Foster City, California) or GeneTool (Double Twist Inc., 2000) with manual adjustments where necessary. Sequences were compared with those from GenBank, via a BLAST search (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 2.3 *Endothiella* isolates (A-H) and location of collection within the *Eucalyptus globulus* plantations surveyed in southwestern Australia and the origin of the *Cryphonectria cubensis* isolates used in the morphological studies.

Isolate Code	Region Isolated	Plantation Number (Figure 2.1)
A2	Albany	22
A17		22
A37		23
A83		21
D32		19
D42		20
B60	Manjimup	9
E10		7
E51		7
E69		11
E81		11
E98		8
F9	Esperance	25
F17		25
G2	Denmark	14
G38		13
G62		17
G75		16
G77		15
H12	Margaret River	6
H15		6
H18		6
H25		5
H29		5
H46		4
H51	Bunbury	2
H57		2
H74		1
H77		1
H118		3
CRY A (DCE 383) CRY B (DCE 384)	*Cryphonectria cubensis*	Collected by E. Davison from *Eucalyptus marginata* in WA

RESULTS

Canker incidence, identification and observations

Approximately 25% of trees examined during the preliminary surveys of two sites within the Albany area (Plantations 19 and 22) had trunk or branch cankers, with *Endothiella* the fungus most commonly associated with these cankers (Table 2.4). Other genera of fungi isolated from cankered tissue were *Botryosphaeria*, *Cytospora*, *Pestalotiopsis*, *Chaetomium, Nigrospora, Epicoccum, Geotrichium,* and *Ulocladium,* of which *Cytospora* was the most common. *Cytospora* was isolated from wood and bark

material separately and often in association with *Endothiella* (Barnett and Hunter, 1998; Hanlin, 1990; Sutton, 1980).

In the larger survey, *Endothiella* was the most frequently recovered fungus from trunk and branch cankers collected from 2450 *E. globulus* trees. *Endothiella* was collected from 21 of the 26 plantations surveyed (Table 2.5). Of the 303 cankers collected, *Endothiella* was isolated from 161. Therefore, 6.6% of all *E. globulus* trees surveyed had *Endothiella* associated with cankers. Bunbury had the highest (15.7%), and Esperance the lowest incidence (1.5%) of trees exhibiting cankers that were associated with the presence of *Endothiella* in the six regions surveyed (Figure 2.3). Canker damage throughout the estate was classified as 'slight', with less than 5% of the trees with 'moderate' canker damage. There was no evidence of 'severe' cankers which would result in the death of the tree.

There was no association between incidence of *Endothiella* in plantations and the environmental conditions at the site, the *E. globulus* provenance, tree form (data not shown), soil type, general tree health (i.e. level of foliage loss due to MLD or insects) or land-use history (Table 2.1). Plantation 17, located in the Denmark region, had a higher incidence of cankers than the other plantation in that region. At this site, defoliation due to *Mycosphaerella* leaf disease (MLD) was 80% or greater. However, defoliation due to MLD or insects (Table 2.1) was not consistently a factor associated with high canker incidence (Table 2.5). In contrast, *Endothiella* was not observed in plantations 15 and 18, also located in Denmark. Trees at these plantations appeared healthy with minimal MLD, but were slower growing compared to plantations of a similar age in that region. Plantation 18 was another site where cankers were not observed. At this plantation, tree form was generally poor with dense bracken-fern (*Pteris* sp.) undergrowth, which may have altered the conditions around the base of the *E. globulus* trees, creating conditions less conducive to canker fungi (Table 2.1). In addition, *Endothiella* was not isolated from plantation 26 in the Esperance region, which is a part of the newer plantation estate. Multi-stem tree form did not necessarily predispose a tree to invasion by *Endothiella* or any other canker-causing pathogen. When cankers were recorded on multi-stemmed trees they were basal cankers. Three cankers isolated from plantation 19, located in the Albany region, were associated with insect damage due to borers (such as *Porocantha* spp.). Plantations which exhibited copper deficiency symptoms did not have an increased canker incidence (Table 2.1 and 2.5).

Table 2.4 Isolation and preliminary identification of fungi recovered from trunk and branch cankers of *Eucalyptus globulus* in two plantations in the Albany region.

Taxon	Frequency of recovery from cankered tissue		Characteristics
	Site 1	Site 2	
Endothiella	92%	69%	White mycelia with orange/brown pycnidia, hyaline conidia cresent-shaped approx 2.5 - 4.5 µm in length; pathogenic or saprophytic on plants.
Cytospora	33%	54%	White-green/brown mycelium; brown-black pycnidia; conidiophores slender; conidia hyaline, elongate-curved (allantoid), 1-celled, approx. 0.2-0.4 µm in length; parasitic, or saprophytic on wood.
Botryosphaeria	0%	8%	Green/brown mycelium; black fruiting bodies; asci bitunicate 8 spored; ascospores hyaline and 1-celled.
Pestalotiopsis	8%	15%	Clear-white mycelium; large brown-black fruiting bodies; asci cylindrical to clavate, short-stipitate, unitunicate, 8-spored; ascospores ovoid to ellipsoidal or fusiform, usually 2-septate, brown, hexagonal or circular in end; approx. 2 µm in length.
Chaetomium	8%	8%	Clear/white sparsely growing mycelia; black fruiting structures; asci 4-8 spored; ascospores 1-celled; brown with germ pore at both ends.
Nigrospora	16%	0%	Grey/brown mycelia, black fruiting structures 1.4 x 1 µm; conidiophores short, mostly simple; conidia (aleuriospres) shiny black, 1-celled, globose, situated on flattened, hyaline (cell) at the end of the conidiophore; parasitic on plants or saprophytic.
Geotrichium	8%	0%	White mycelium; conidiophores absent; hyaline conidia (arthrospores), 1-celled, short cylindrical with truncate ends, formed by segmentation of hyphae; mostly saprophytic, common in soil.
Ulocladium	0%	23%	Cream/brown mycelia with brown spores 2 µm in length; conidiophores indeterminate, sympodial, dark, mostly simple, septate; conidia (porospores) dark, dictyosporous, borne singly, apical and new sympodial growing points; saprophytic.
Epicoccum	8%	0%	Orange/brown mycelium; orange fruiting bodies approx 2.4 µm in length; conidiophores compact or loose, dark; conidia dark, several celled (dictyosporous), globose approx. 0.8 x 0.25 µm; mostly saprophytic, or weakly parasitic.

Measle cankers, located on trunks and lateral branches, were frequently superficial, did not reach the cambium and were often contained and shed by the host (Figure 2.2A). *Endothiella* was isolated from measle cankers collected from Plantation 13 in the Denmark region (from four of five measle cankers sampled) and Plantation 7 near Manjimup.

Basal cankers were more common than measle cankers and were frequently associated with *Endothiella.* Symptoms of basal canker infection included swelling of the basal region of the trees, a surface darkening of stem tissue, lesions extending into wood (5-10 mm) and kino exudation (Figure 2.2B and C). Small orange pycnidia were often associated with basal cankers. Pycnidia were also present on stem or bark material in the absence of a lesion (Figure 2.4). At the time of sampling, basal cankers did not appear to influence the overall health of the tree. There was no sign of wilt or dying back of the top of the tree which would be associated with stem girdling. Kino exudation associated with basal cankers was rare (only two instances of kino exudation recorded).

Four of the trees surveyed at plantation 13 (located near Denmark) exhibited symptoms of 'tip dieback'. *Endothiella* was recovered from two of these samples and *Cytospora* from one other. Tip dieback was recorded only in the Denmark region.

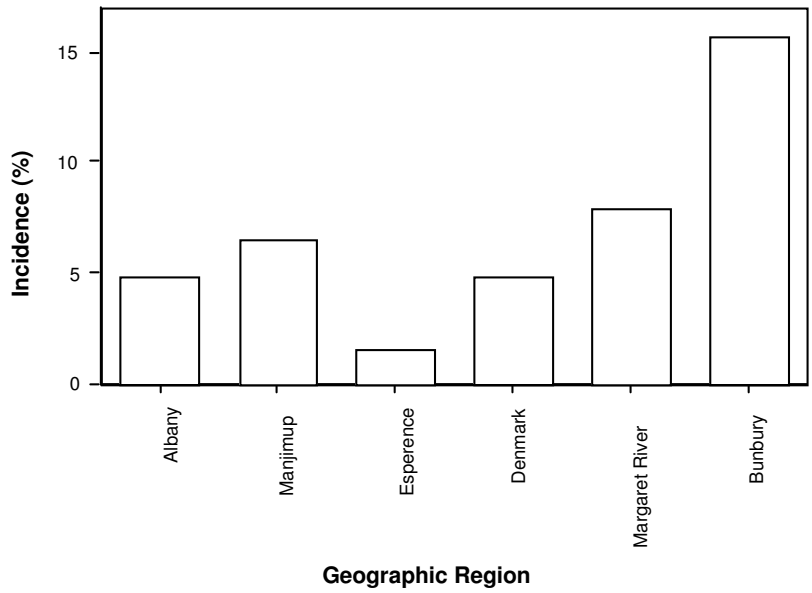


Figure 2.3 Mean incidence of *Endothiella* recovered from cankers in two- and three-year-old *Eucalyptus globulus* plantations in southwestern Australia.

Figure 2.4
Characteristic orange pycnidia of *Endothiella* on the bark surface *Eucalyptus globulus*. Bar = 5 mm

Table 2.5 Incidence of cankers associated with *Endothiella* in *Eucalyptus globulus* plantations surveyed in the six regions of southwestern Australia.

Location	Site#	No. trees sampled with cankers Transect 1	Transect 2	Incidence of *Endothiella* (%)	*Endothiella* isolated from cankers (%)
Albany	19	8	6	0	0
	20	3	7	8	80
	21	5	3	2	25
	22	5	6	11	100
	23	2	NA	2	100
	24	1	4	3	60
Bunbury	1	5	8	7	54
	2	7	7	11	78
	3	18	24	29	69
Denmark	12	6	NA	3	100
	13	11	7	7	39
	14	6	4	3	30
	15	0	NA	0	0
	16	2	4	4	66
	17	3	9	10	83
	18	0	0	0	0
Esperance	25	12	5	3	18
	26	1	1	0	0
Manjimup	7	6	17	13	56
	8	5	4	4	44
	9	8	7	5	33
	10	2	4	0	0
	11	2	12	9	64
Margaret River	4	2	2	2	25
	5	15	19	20	59
	6	4	4	2	25

***Microscopic identification of* Endothiella**

Examination of conidia and conidiophores of the *Endothiella* isolates collected in the current study enabled the fungus to be identified as the anamorph of *Cryphonectria eucalypti*. This was based on the following characteristics - stromata erumpent through bark of host, pulvinate, bright orange to chestnut, 1.5-3 mm in diameter by 1.5-2 mm in length (Figure 2.5); conidia one-celled, hyaline, cylindric to allantoid, generally 2.5 - 4.5 µm in length, and 0.9 - 1.3 µm in breadth, formed in locules in young stromata (Figure 2.6).

Figure 2.5 Orange pycnidia of *Endothiella* on the bark of a basal canker. Bar = 2 mm.

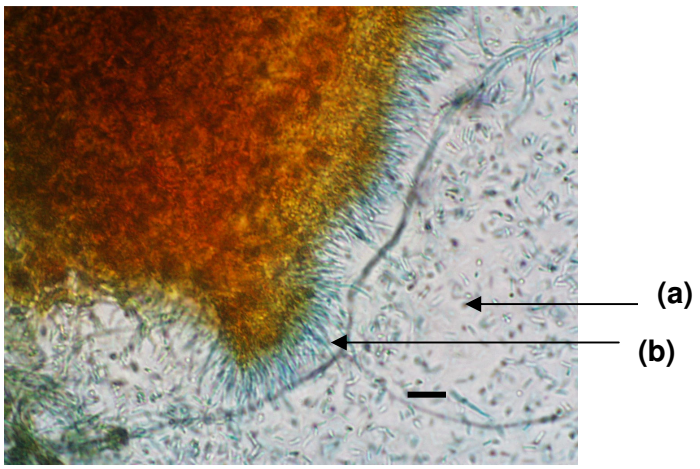


Figure 2.6 Conidia (a) and conidiogenous cells (b) of *Endothiella eucalypti* isolated from cankers on *Eucalyptus globulus* in plantations throughout southwestern Australia. Bar = 10 µm.

Molecular analysis

The results of sequencing *Endothiella* isolates and comparison using a BLAST search confirmed the taxonomic identification as *En. eucalypti*. *Endothiella eucalypti* isolates of the current study match with accuracy (98-99%) the isolates of *Endothia gyrosa* (now *C. eucalypti*) as listed in GenBank. Therefore, the anamorph collected during the current study will be referred to as *En. eucalypti* throughout this thesis.

Macromorphology

A colony morphology classification system was developed (Figure 2.7; Table 2.6) based on colony characteristics of the fungus when grown on 1/2PDA such as: mycelium colour, mycelium growth pattern and pycnidia distribution. The 30 *En. eucalypti* isolates and two *C. cubensis* isolates were assessed according to this system and assigned to one of the 10 morphological growth types (Table 2.6).

A selection of isolates (A2, H12, H18, H29 and H74) sub-cultured from the same single spore parent culture developed distinctly different colony morphologies (Figure 2.8). The colony morphology of the two *C. cubensis* isolates was distinctly different from the *En. eucalypti* isolates.

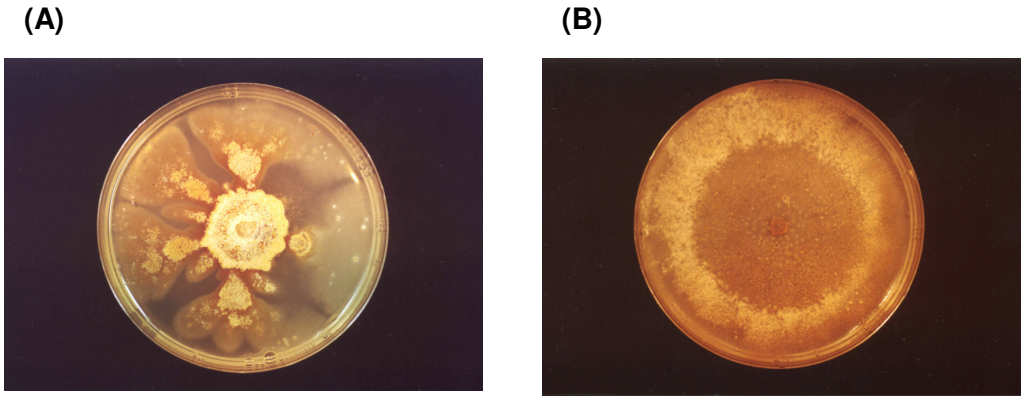
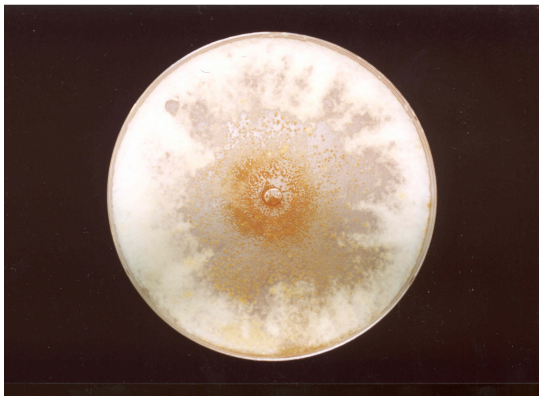


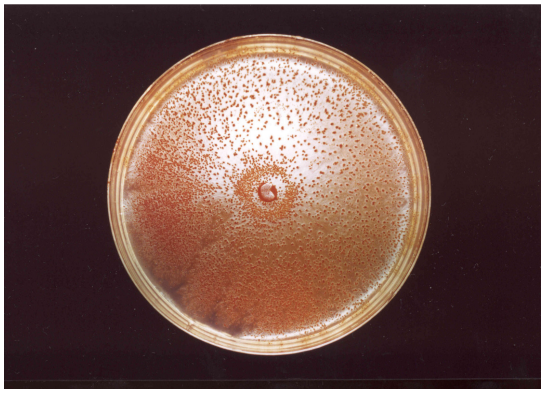
Figure 2.8 Two different colony morphologies obtained from the same single spore culture of *Endothiella eucalypti* isolate H29; Type A and Type B.

Type 1

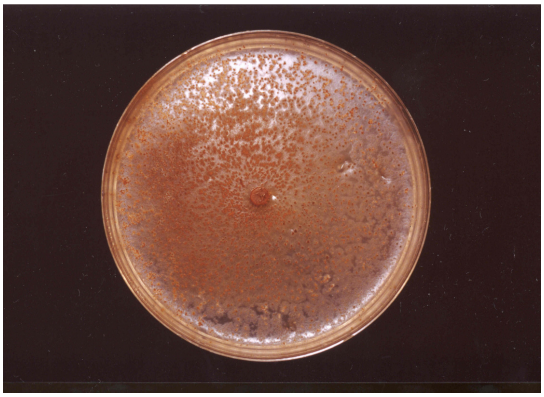


Type 2

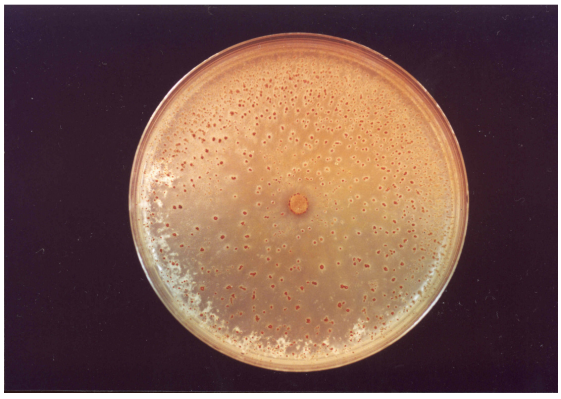
Type 3



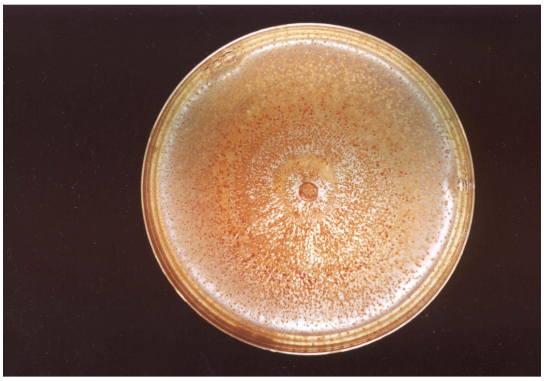
Type 4



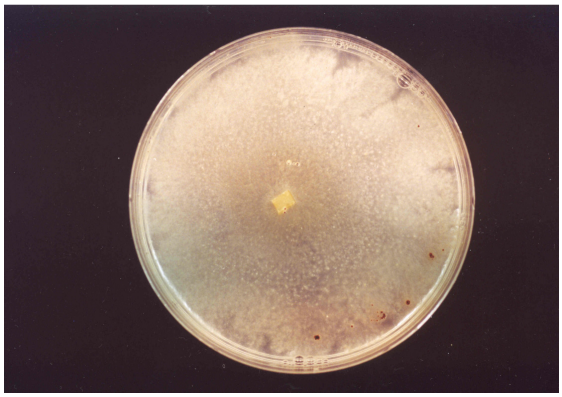
Type 5



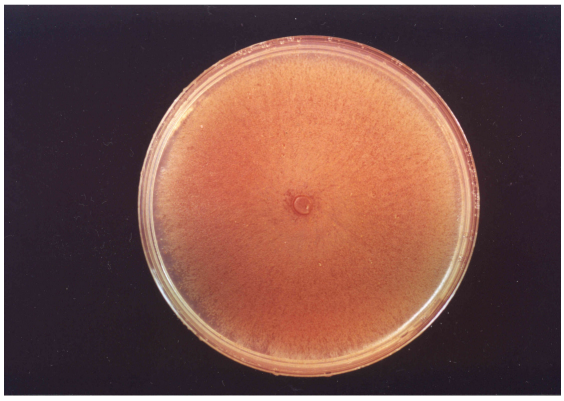
Type 6



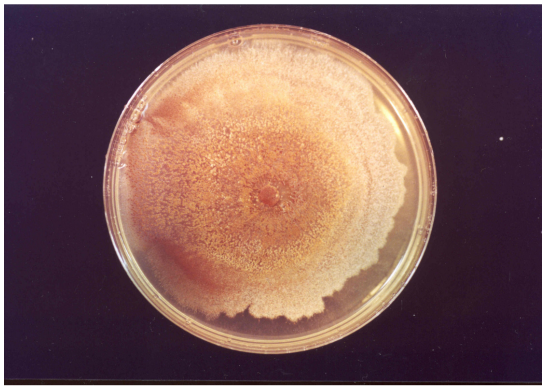
Type 7



Type 8



Type 9



Type 10

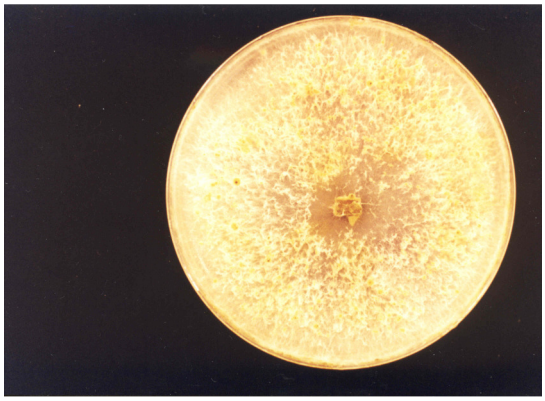


Figure 2.7
Ten colony morphotypes of *Endothiella eucalypti and Cryphonectria cubensis*. See Table 2.6 for descriptions.

Table 2.6 Grouping of *Endothiella eucalypti* and *Cryphonectria cubensis* isolates according to colony morphology when grown at 25 ℃ for one month on 1/2 strength potato dextrose agar.

Characteristic* **Isolate**	**1. Mycelium colour**	**2. Mycelium pattern**	**3. Pycnidia**	**4. Pycnidia colour**	**5. Pycnidia pattern**	**Morphology type**
A2 (type A)	a	f	a	a	c	1
H12(type A)	a	f	a	a	c	1
H18 (type B)	a	f	a	a	c	1
H25	a	f	a	a	c	1
H29 (type B)	a	f	a	a	c	1
H46	a	f	a	a	c	1
H51	a	f	a	a	c	1
H57	a	f	a	a	c	1
H74 (type A)	a	f	a	a	c	1
A17	a	b	a	a	d	2
A37	a	b	a	a	d	2
B60	a	b	a	a	d	2
E51	a	b	a	a	d	2
H29 (type A)	a	b	a	a	d	2
A2 (type B)	b	e	a	a	a	3
D42	b	e	a	a	a	3
E69	b	e	a	a	a	3
E81	b	e	a	a	a	3
E10	b	e	a	a	c	4
F17	b	e	a	a	c	4
G2	b	e	a	a	c	4
G77	b	e	a	a	c	4
H12 (type B)	b	e	a	a	c	4
H15	b	e	a	a	c	4
H18 (type A)	b	e	a	a	c	4
H74 (type B)	b	e	a	a	c	4
D32	a	f	a	a	a	5
E98	a	f	a	a	a	5
G62	a	f	a	a	c	5
H118	a	f	a	a	a	5
A83	a	a	a	a	b	6
F9	a	i	a	a	c	6
G38	a	g	a	b	e	7
G75	c	c	b	na	na	8
H77	a	h	a	a	c	9
CRY A	a	d	a	b	e	10
CRY B	a	d	a	b	e	10

***Characteristic**			
1. Mycelium colour	**(a)** white	**(b)** colourless	**(c)** orange
2. Mycelium pattern	**(a)** ringed **(e)** smooth **(h)** crusty	**(b)** flower-shaped **(f)** smooth with fluffy edge **(i)** tufts	**(c)** linear **(g)** feathered
3. Pycnidia	**(a)** present	**(b)** absent	
4. Pycnidia colour	**(a)** orange	**(b)** other	
5. Pycnidia pattern	**(a)** scattered **(d)** random clusters	**(b)** ringed **(e)** individual	**(c)** uniform

*Morphology type based on characteristics 1-5.

DISCUSSION

The most frequently isolated fungus associated with cankers in *E. globulus* plantations in southwestern Australia was identified by classical and molecular techniques to be *Endothiella eucalypti,* the anamorph of *Cryphonectria eucalypti*. The morphological characteristics used for the identification of *En. eucalypti* were consistent with previous descriptions outlined by Ventor *et al.* (2002); Roane *et al.* (1986), Walker *et al*. (1985), Sutton (1980), Barr (1978) and Snow *et al*. (1975). In addition, the ITS sequence data for *En. eucalypti* had greater than 98% homology with isolates of *End. gyrosa* (now *C. eucalypti*) in GenBank. The two methods thus established that the most common pathogen of *E. globulus* isolated in the current study was *En. eucalypti*.

Endothiella eucalypti was relatively widespread in the WA plantation estate, however, the severity of disease on healthy *E. globulus* was low. *Endothiella eucalypti* pycnidia were often isolated from the outer bark of two- to three-year-old *E. globulus* saplings, however there was no evidence of pathogen invasion causing significant disease or tree death. Other fungi recovered from cankers, in association with or separate from *En. eucalypti,* included *Botryosphaeria* and, more commonly, *Cytospora*. These fungi have previously been isolated from diseased eucalypts. For example, *Botryosphaeria ribis* has previously been identified as the causal agent of cankers in young *E. globulus* in WA (Morgan, 1994) and *Cytospora eucalypticola* is commonly recovered from diseased tissue of eucalypts in WA (Davison and Tay, 1983). However, *Endothiella eucalypti* was identified as the most likely fungus to cause cankers in eucalypts in southwestern Australia (Tovar, 1998; Fraser and Davison, 1985; Davison and Tay, 1983).

Although it has been reported from southeastern Australia (Walker *et al.*, 1985) and Tasmania (Yuan and Mohammed, 1997), *C. eucalypti,* the teleomorph of *En. eucalypti*, was not observed in the current study and has not previously been found in southwestern Australia (Davison, 1995; Shearer, 1994). *Endothiella eucalypti* was considered native to southwestern Australia (Davison and Tay, 1983), however due to the absence of the teleomorph evidence for place of origin has been questioned (Shearer, 1994). Therefore, the life-cycle of this fungus in southwestern Australia is currently unknown.

Cytospora was frequently isolated from diseased eucalypts in the current study and in previous studies, however it has been shown to be a non-aggressive, opportunistic

coloniser of eucalypts (Old *et al.*, 1986; Fraser and Davison, 1985; Davison and Tay, 1983). In the current study, *Cytospora* was often isolated from cankered material in association with *En. eucalypti*. This has been reported previously by Davison and Tay (1983) who isolated *Cytospora* independently, as well as associated with other fungi, from older cankers. It was this observation that led the authors to suggest that *Cytospora* invades after or in association with *En. eucalypti* and gradually replaces it. Evidence from the current study and those undertaken previously (Davison and Tay, 1983) indicates that *En. eucalypti* appears to be the first pathogen to invade *E. globulus* thereby creating an environment for other less aggressive fungi to colonise.

The incidence of cankers caused by *En. eucalypti* was higher in some regions with a longer history of *E. globulus* plantations, such as Bunbury, compared to Esperance, the most recently planted region, which had the lowest canker incidence. Therefore, it is likely that the incidence of disease caused by canker-fungi within the plantation environment will continue to increase as the estate ages. This finding is particularly important in the management of canker disease in plantations which are left to coppice after harvest, as this practice exposes the newly sprouting stems to an increased inoculum potential from the previous plantation.

The term 'tip dieback' describes a condition in which the tree dies from the tips of the branches down the tree. This condition has often been associated with multi-stemmed trees (Hardy *pers. comm.*). A survey of eucalypt plantations in South Africa during 1991 and 1992 showed widespread twig dieback on a variety of eucalypt species. Trees most commonly affected were aged between one and two years (Smith *et al*., 1994). In the current study, this condition was only recorded in the Denmark region where its effects were severe. However, *En. eucalypti* was infrequently recovered from 'tip dieback' material, and further investigations are required to establish the causal agent of this condition.

There was no clear link between morphological classification and collection location, however, the use of macromorphological characteristics did separate the *En. eucalypti* isolates from the *C. cubensis* isolates. Further molecular studies to establish whether the macromorphological variation within the southwest population of *En. eucalypti* indicate variation at a DNA level are reported in Chapter 6.

Although *En. eucalypti* was widespread throughout the *E. globulus* plantation estate of southwestern Australia, it (and other canker-causing fungi) are not currently seen as a

major threat to the health of plantations in the region. However, there is great potential for dissemination of these fungi within and between plantations and it is likely that disease incidence will increase as the plantation estate ages. In studies reported in Chapters 3 and 4 the pathogenicity of *En. eucalypti* isolates collected from the current survey and the susceptibility of a range of *E. globulus* provenances were examined in the glasshouse and in the field.

Chapter 3

Phenotypic variation in *Endothiella eucalypti* isolated from *Eucalyptus globulus* plantations in southwestern Australia

INTRODUCTION

Endothiella eucalypti was the fungus most commonly recovered from stem cankers in two- to three-year-old *Eucalyptus globulus* plantations in southwestern Australia (Chapter 2). The pathogenicity of a small number of WA isolates of *En. eucalypti* has previously been established in coppiced stems of *E. marginata* (Davison and Tay, 1983) and *E. saligna* stems (Fraser and Davison, 1985). More recent studies on two-year-old *E. globulus* saplings indicated that *En. eucalypti* was more pathogenic to this host than *Botryosphaeria ribis* (Shearer and Fairman, unpubl). This study also reported a variation in the pathogenicity of *En. eucalypti* to *E. globulus* and other eucalypt species (Shearer and Fairman, unpubl). Previous studies in eastern Australia and Tasmania have also indicated a variation in pathogenicity of *En. eucalypti* and its teleomorph in a number of eucalypt species (Old *et al.*, 1990; Wardlaw, 1999; Yuan and Mohammed, 2000). The teleomorph, *Cryphonectria eucalypti* has been shown to cause significant lesions on *Eucalyptus* clones in the field in South Africa (Gryzenhout *et al.*, 2003). Again, a variation in pathogenicity between *C. eucalypti* isolates was recorded (Gryzenhout *et al.*, 2003). Whilst *En. eucalypti* and its teleomorph are generally considered opportunistic pathogens of healthy eucalypts in Australia (Old *et al.*, 1990; Davison, 1995), the potential of *En. eucalypti* to cause disease in WA *E. globulus* plantations has not yet been established.

Venter *et al.* (1999) compared the mycelial growth rates of *Endothia gyrosa* (now *C. eucalypti* for isolates from Australia and South Africa) isolates collected from South Africa, Australia and North America incubated at temperatures from 10 to 30 °C *in vitro*. The results showed that the growth rate of the fungi *in vitro* separated the *End. gyrosa* isolates into two distinct groups. The South African and Australian isolates exhibited similar growth rates at the various temperatures, whereas the isolates originating from North America were slower growers. Recent molecular studies have since separated the South African and Australian isolates from the North American isolates and the fungus from South Africa and Australia has been re-named *C. eucalypti* (Venter *et al.*, 2002). Therefore, variations in the response of fungal isolates to

temperature *in vitro* may be useful in grouping isolates according to their place of origin.

Environmental conditions in the field have been shown to influence the pathogenicity of *C. eucalypti* to *Eucalyptus* clones (Gryzenhout *et al.*, 2003). Temperature has been identified as a factor which significantly influences the pathogenicity of a pathogen to its host. For example, Huberli *et al.* (2002) reported that an increase in temperature from 20 to 30 °C resulted in an increase in lesion length in excised *E. marginata* stems after inoculation with *Phytophthora cinnamomi*. Therefore, it is important to establish and be aware of the conditions which favour the success of a pathogen.

The study reported in this chapter was designed to determine the pathogenicity and temperature response of *En. eucalypti* collected in Chapter 2 by:

(i) examining the pathogenicity of the *En. eucalypti* isolates under glasshouse conditions;

(ii) investigating the *in vitro* mycelial growth rate of *En. eucalypti* isolates at 15, 20, 25 and 30 °C; and

(iii) determining the effect of temperature on the ability of *En. eucalypti* to cause disease in excised *E. globulus* stems.

MATERIALS AND METHODS

Pathogenicity of *Endothiella eucalypti*

Experimental design

The pathogenicity of 29 *En. eucalypti* isolates to one-year-old *E. globulus* saplings (average height 1 m, with average stem diameter of 9 mm at the point of inoculation 200 mm above the base of the stem) was investigated using a randomised complete block design in the glasshouse (Steel and Torrie, 1986). Five saplings were used for each of the *En. eucalypti* isolates and another five as controls. One stem from each plant was inoculated with an *En. eucalypti* isolate or a sterile Miracloth® (Calbiochem, Victoria, Australia) disc, as a control. Lesion extension and pathogen colonisation of the stem were determined three weeks after inoculation.

Biological materials

Endothiella eucalypti isolates associated with *E. globulus* cankers (Chapter 2) were used. Five isolates were selected at random from each of the Albany, Bunbury and Denmark regions; six isolates were selected from the Margaret River and Manjimup

regions and two isolates from the Esperance region (Table 3.1). The 29 isolates were maintained on 1/2 PDA (Chapter 2) at 24 °C under continuous near blue light (NEC T10 Blacklight).

To ensure that all isolates were of similar physiological status prior to use, they were passaged through excised *E. globulus* branches. Briefly, each isolate was inoculated into an excised two-year-old *E. globulus* branch (average diameter of 10 mm, approximately 50 cm in length), using the underbark method of inoculation (see 'Inoculation' section below) and the branch incubated at 25 ˚C for 7-14 days. After this time, the inoculated branch was surface sterilized with 70% ethanol, briefly flamed, plated onto 1/2PDA+S plates (Chapter 2) and incubated at 24 ˚C in the dark. *Endothiella eucalypti* was then sub-cultured from these plates onto 1/2PDA plates, where they were maintained at 24 ˚C under continuous near blue light (7-10 days) prior to inoculum preparation.

The inoculum was prepared by sub-culturing five, 5 mm diameter agar pieces from actively growing colonies of each of the *En. eucalypti* isolates onto 1/2PDA covered with sterile Miracloth® discs (5 mm diameter) and incubating them for seven days at 24 ˚C under continuous near blue light (Figure 3.1). The inoculum discs contained a mixture of spores and mycelium. The Miracloth® discs had previously been sterilised by autoclaving for 20 minutes at 121 °C on three consecutive days prior to placing on the agar. Sterile non-colonised discs were used as controls.

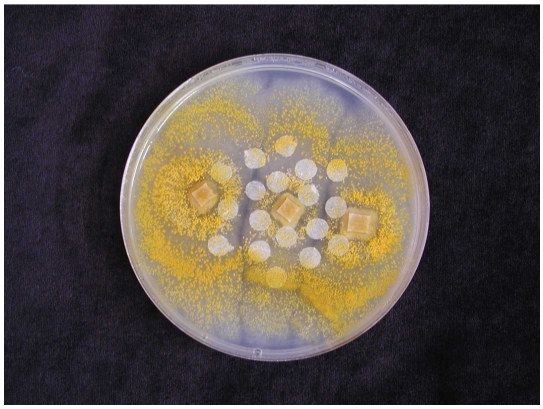


Figure 3.1
Half-strength potato dextrose agar plate containing sterile Miracloth® discs covered in *Endothiella eucalypti.*

One-year-old *E. globulus* saplings, supplied by Integrated Tree Cropping Ltd (ITC, Albany, WA), were grown in free-draining 150 mm diameter pots containing potting mix in an evaporatively-cooled glasshouse (20-27 ˚C min-max) in late spring (October). Potting mix composition included 2:2:1 coarse river sand; composted pine bark fines;

cocoa peat (coir dust) (SSM1580; Richgro, Western Australia) with additions of Dolomite (1.5 g/ L potting mix), calcium carbonate ($CaCO_3$; 1.5 g/L potting mix) and six month slow-release fertiliser (Scotts Osmocote Plus, Scotts Europe BV, Heerlen, The Netherlands; 1 g/L potting mix). Saplings were watered via an overhead watering system for 20 minutes twice a day and fertilised by hand with Wuxal® Liquid Foliar Nutrient (Hoechst Schering AgrEvo Pty Ltd, 1731-1733 Malvern Rd Glen Iris Victoria, Australia) once a week (10 mL/10L water) for two months prior to the commencement of the trial.

Inoculation

The main stem of intact sapling was inoculated under the bark 200 mm above the soil line with an *En. eucalypti* colonised disc. Briefly, a sterile blade was used to cut a shallow incision through the periderm to the phloem (approximately 7-10 mm in length longitudinally) with care taken to avoid damage to the xylem. A colonised disc was then placed into the incision (mycelium side against stem), the wounded stem wrapped in Parafilm (American National Can, Chicago, USA) and again with flagging tape to avoid fungal desiccation and light penetration. Sterile non-colonised Miracloth® discs were inserted into stems as controls using the same procedure as for colonised discs.

Harvest

At harvest, lesion extension was measured and stems then cut into seven 10 mm segments up the stem. The first segment contained the lesion front and the remaining segments extended 60 mm beyond the lesion front. Each 10 mm stem segment was cut longitudinally and surface sterilised by dipping stem sections into 70% ethanol, briefly flamed (1-2 sec) and then plated onto 1/2PDA+S. Fungal growth was monitored from each of the segments over seven days and total colonisation of the stem by *En. eucalypti* beyond the lesion determined (Figure 3.2). To determine whether the inoculum source was still viable at the time of harvest, the inoculum discs were recovered from each stem and plated onto 1/2PDA+S.

Figure 3.2
Eucalyptus globulus stem segments placed onto half-strength potato dextrose agar containing streptomycin to determine colonisation of *Endothiella eucalypti* beyond the lesion front.

Table 3.1 *Endothiella eucalypti* isolates used in pathogenicity and mycelial growth rates studies.

Isolate Code	Region Isolated	Plantation Number (Figure 2.1)	Pathogenicity	Temperature
A2	Albany	22	√	√
A17		22	√	√
A37		23	x*	√
A83		21	√	√
D32		19	√	√
D42		20	√	x*
B60	Manjimup	9	√	√
E10		7	√	√
E51		7	√	√
E69		11	√	x
E81		11	√	√
E98		8	√	√
F9	Esperance	25	√	√
F17		25	√	√
G2	Denmark	14	√	√
G38		13	√	√
G62		17	√	√
G75		16	√	√
G77		15	√	√
H12	Margaret River	6	√	√
H15		6	√	√
H18		6	√	√
H25		5	√	√
H29		5	√	√
H46		4	√	x
H51	Bunbury	2	√	√
H57		2	√	x*
H74		1	√	√
H77		1	√	√
H83		3	x*	√
H118		3	√	√

*Isolates A37 and H83 were replaced with D42 and H57 as the former were not successfully passaged prior to commencement of the trial.

KEY
√ = present in trial
x = not present in trial

Temperature response of *Endothiella eucalypti in vitro*

Experimental design

The mycelial growth rate of 27 *En. eucalypti* isolates at 15, 20, 25 and 30 °C was determined, with three replicate plates for each isolate at each temperature. Plates were incubated for seven days after which time radial growth and mycelial dry weight were recorded.

Endothiella eucalypti *isolates*

The isolates used in the glasshouse pathogenicity trial were examined for *in vitro* growth, with the exception of isolates A37 and H83 which were replaced with D42 and H57, respectively, and isolates E69 and H46 which were omitted to maintain the five replicate isolates from each region (Table 3.1). The 27 isolates were maintained on 1/2PDA at 24 °C under continuous near blue light.

Petri-dishes (9 cm diameter) containing 1/2 PDA were completely covered with sterile cellophane discs. Briefly, cellophane discs (Hallmark, Australia) were boiled twice, for one hour each time, in deionised water containing EDTA (100 µg/ 10 L). The discs were rinsed in deionised water, placed into glass Petri dishes and autoclaved at 121 °C for 20 min on three consecutive days before being used to cover the 1/2PDA plates. Cellophane-covered plates were centrally inoculated with a 5 mm^2 agar piece from a seven-day-old actively growing colony of each *En. eucalypti* isolate. The isolates were then incubated for seven days at 24 °C in the dark.

At harvest, the diameter of the mycelial colony was measured (for each plate) from three points (120° apart) around the circumference of the colony. The mycelium was then scraped, using a scalpel blade, from the cellophane, placed into a pre-weighed 1.5 mL microfuge tube, dried at 60 °C to a constant weight (48 hours) and the dry weight recorded.

Pathogenicity of *Endothiella eucalypti* in excised *Eucalyptus globulus* stems at four temperatures

Experimental design

Endothiella eucalypti isolate E81 was inoculated into excised stems of *E. globulus* saplings and incubated in moist chambers at 15, 20, 25 and 30 °C for 14 days in the dark. This isolate was originally collected from Manjimup (Chapter 2; Table 3.1) and was identified as the most pathogenic of the 29 isolates screened in the pathogenicity

trial. Six replicate stems were used for each temperature. At harvest, lesion extension and pathogen colonisation of the stems were determined.

Biological materials and methods

Excised branches of four-year-old *E. globulus* saplings were prepared for inoculation according to the method of Huberli *et al*. (2002). Briefly, excised branches were trimmed into 50 cm lengths (averaging 5.5 mm diameter) and side branches removed. Once cut, branch ends were dipped into melted parafin wax to avoid desiccation. Prior to inoculation, the branches were surface sterilised with 70 % ethanol. Excised branches were inoculated under the bark (see previous section on 'Inoculation') with *En. eucalypti* isolate E81 at the centre of each branch and the wound sealed with Parafilm (American National Can, Chicago, USA) to prevent desiccation. Controls were inoculated with sterile Miracloth® discs. Inoculated branches were incubated in the dark at 15, 20, 25 and 30 °C in moist chambers. The chambers consisted of surface sterilised 10 L plastic trays (39 x 27 x 6 cm), lined with moist paper towels and sealed in a plastic bag. Six replicate branches and controls were placed in each of the four containers (one container for each temperature) and stored at their allocated temperature for 14 days. After this time, lesion extension and pathogen colonisation (as outlined previously) were determined.

Data analysis

In order to facilitate comparison between chapters, lesion extension is expressed as mm/week throughout this thesis. Lesion extension (mm/week), colonisation beyond the lesion (mm/week), mycelial radial growth (mm/week) and mycelial dry weight (mg/week) were statistically analysed by Analysis of Variance (ANOVA) using Statistica Version 4.1 (StatSoft® Inc., OK, USA). Data were assessed for homogeneity, variation of the mean from the variance and fit to a normal distribution. Means were compared by Least Significant Difference (LSD) test (at $p \leq 0.05$ for the pathogenicity screening, $p \leq 0.01$ for mycelial growth study and at $p \leq 0.05$ for the pathogenicity of isolate E81 in excised stems) and presented with standard errors of the mean.

RESULTS

Pathogenicity of *Endothiella eucalypti*

There was a significant ($p<0.0001$) difference in lesion extension and total colonisation (lesion extension + colonisation of stem above lesion) between the *En. eucalypti* isolates, with inoculation of all isolates, except F17, resulting in the formation of lesions (Figure 3.3, Tables 3.2 and 3.3). Isolate E81, collected from Manjimup, caused significantly ($p\leq0.05$) larger lesions and colonised the stems of *E. globulus* saplings to a greater extent than 20 and 21 of the 29 isolates, respectively (Figures 3.3 and 3.4). Although isolate F17 did not cause lesions, it was recovered from the Miracloth® disc inoculum and from the stem immediately beneath the disc. All remaining *En. eucalypti* isolates were also recovered from the original inoculum discs removed from the inoculated stems at harvest. Assessment of the extent to which *En. eucalypti* girdled the *E. globulus* stems was attempted repeatedly, this was not successful.

The location of isolate collection had a significant ($p=0.001$ and $p=0.004$) effect on isolate pathogenicity (lesion extension and total colonisation, respectively) (Tables 3.2 and 3.3). Overall, isolates from the Denmark region formed the largest lesions and colonised the stem to a greater extent than isolates from the other five regions. In comparison, the extent of the lesions caused by the Esperance isolates was significantly ($p\leq0.05$) less than lesions resulting from inoculation with isolates from the other five regions (Figure 3.4). There were no trends relating the lesion extension or total colonisation of isolates to the region of isolate collection (Table 3.4 and Figure 3.4). Within a region, isolates had similar sized lesions, with the exception of isolates from the Manjimup and Esperance regions (Figure 3.4).

There was a positive correlation ($r=0.80$) between the total colonisation of the stems (mm/week) by *En. eucalypti* and the extension of the lesion (mm/week) (Figure 3.5). Isolates were separated into four groups on the basis of lesion development (Table 3.4). Where present, lesion extension ranged from 12.5 to 30 mm/week (Table 3.4).

Table 3.2 ANOVA of lesion extension (mm/week) in stems of *Eucalyptus globulus* inoculated with *Endothiella eucalypti*. Significant values are in bold font.

Effect	MS Effect	MS Error	F (df)	P
Location	257.105	54.787	4.693 (5, 124)	**0.001**
Isolate	147.598	39.073	3.777 (28, 101)	**≤0.0001**

Table 3.3 ANOVA of total colonisation (mm/week) in stems of *Eucalyptus globulus* inoculated with *Endothiella eucalypti*. Significant values are in bold font.

Effect	MS Effect	MS Error	F (df)	P
Location	441.653	119.180	3.706 (5, 124)	**0.004**
Isolate	300.223	84.954	3.534 (28, 101)	**≤0.0001**

Figure 3.3
Example of a lesion caused by underbark inoculation of *Eucalyptus globulus* stems with *Endothiella eucalypti* isolate E81 (bottom) compared to a non-inoculated stem (top). Bar = 1 cm.

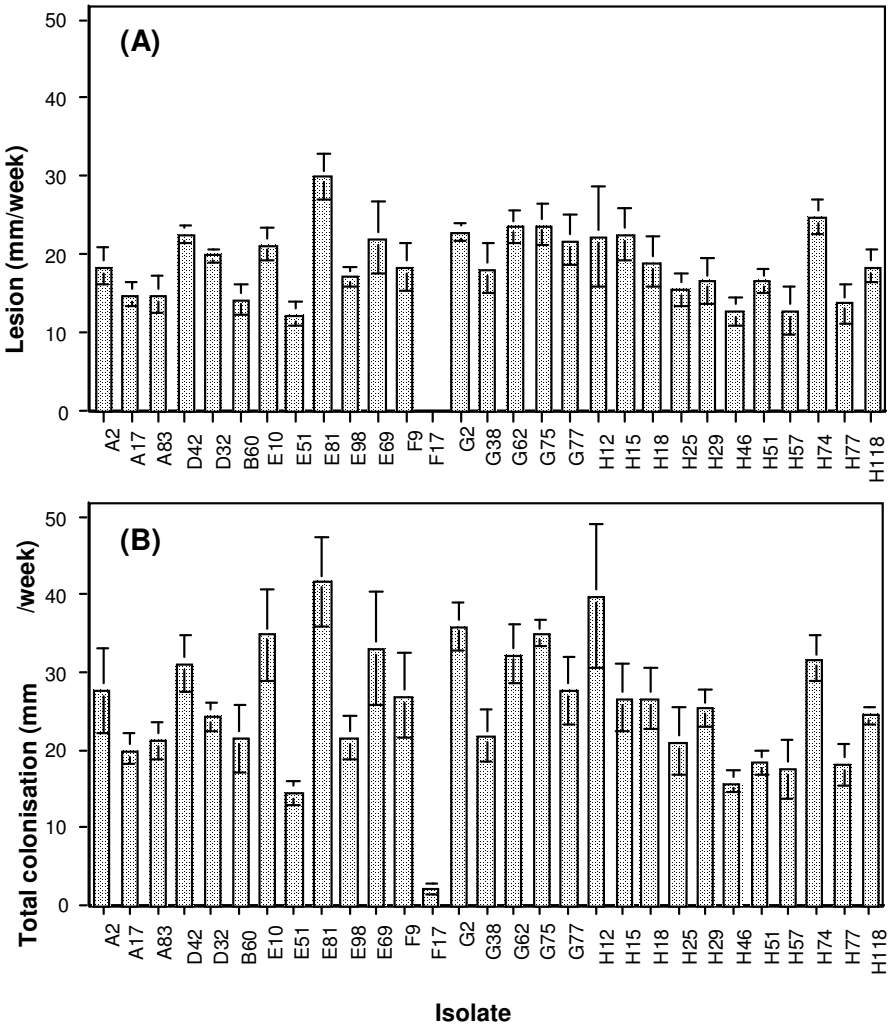


Figure 3.4 (A) Lesion extension (mm/week) and (B) total colonisation (mm/week) (±SE) in stems of one-year-old *Eucalyptus globulus* seedlings inoculated with *Endothiella eucalypti* isolates collected from the Albany (A2, A17, A83, D42, D32), Manjimup (B60, E10, E51, E81, E98, E69), Margaret River (H12, H15, H18, H25, H29, H46), Bunbury (H51, H57, H74, H77, H118), Denmark (G2, G38, G62, G75, G77) and Esperance (F9, F17) regions of southwestern Australia.

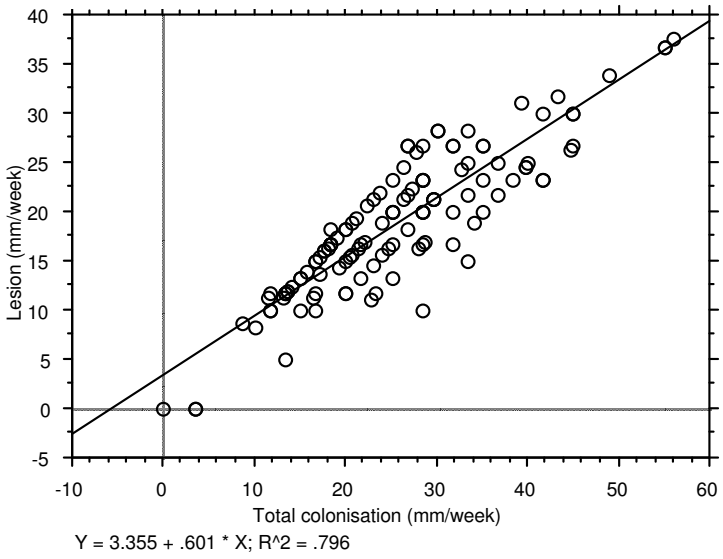


Figure 3.5 Correlation between lesion extension (mm/week) and total colonisation (mm/week) in stems of one-year-old *Eucalyptus globulus* saplings inoculated with *Endothiella eucalypti* isolates collected from Albany, Manjimup, Margaret River, Bunbury, Denmark and Esperance regions of southwestern Australia.

Table 3.4 Ranking according to lesion extension (mm/week) in stems of one-year-old *Eucalyptus globulus* saplings inoculated with *Endothiella eucalypti* isolates collected from southwestern Australia.

Classification group	Isolate number	Mean lesion ± SE (mm/week)	Isolate collection location	Observations
Non pathogenic	F17	0 ± 0	Esperance	Callus forming behind point of inoculation.
Weakly pathogenic	E51	12.53 ± 1.64	Manjimup	PYC on 20% of stems
	H46	12.92 ± 1.72	Margaret River	PYC on 40% of stems Gummosis on 40% of stems
	H57	12.92 ± 3.18	Bunbury	PYC on 60% of stems
	H77	13.83 ± 2.5	Bunbury	PYC on 20% of stems
	B60	14.42 ± 1.94	Manjimup	PYC on 60% of stems
	A17	15 ± 1.58	Albany	
	A83	15 ± 2.22	Albany	PYC & gummosis on 40% of stems
	H25	15.67 ± 2.16	Margaret River	PYC on 60% of stems 20% wilted
	H29	16.67 ± 2.93	Margaret River	40% stems wilted PYC & gummosis on 20% of stems
	H51	16.75 ± 1.62	Bunbury	
	E98	17.33 ± 1.36	Manjimup	PYC on 40% of stems
	G38	18.42 ± 3.21	Denmark	PYC on 40% of stems Gummosis or wilting on 20% of stems
Moderately pathogenic	A2	18.6 ± 2.42	Albany	PYC on 60% of stems 40% of stems wilted
	H118	18.67 ± 2.16	Bunbury	
	F9	18.67 ± 3.09	Esperance	PYC on 80% of stems 20% dried leaves
	H18	19.17 ± 3.23	Margaret River	PYC on 20% of stems
	D32	20 ± 0.91	Albany	PYC on 80% of stems Gummosis or dried leaves on 20% of stems
	E10	21.5 ± 2.05	Manjimup	
	G77	22 ± 3.14	Denmark	PYC on 100% of stems Gummosis or wilting on 20% of stems
	E69	22.25 ± 4.54	Manjimup	PYC on 100% of stems
	H12	22.42 ± 6.40	Margaret River	PYC on 100% of stems 20% wilted/dried
	H15	22.58 ± 3.32	Margaret River	PYC on 80% of stems Gummosis on 20% of stems
	D42	22.75 ± 1.02	Albany	PYC on 40% of stems Gummosis on 20% of stems
	G2	23 ± 1.11	Denmark	PYC on 100% of stems 40% dried leaves Gummosis on 20% of stems
	G62	23.67 ± 2.13	Denmark	PYC on 100% of stems 40% dried leaves
	G75	24 ± 2.51	Denmark	PYC on 100% of stems 20% dried leaves
	H74	25 ± 2.24	Bunbury	PYC on 100% of stems 80% dried leaves
Strongly pathogenic	E81	30 ± 2.84	Manjimup	PYC on 100% of stems 80% dried leaves = dead

Key

PYC = orange pycnidia

Gummosis = observed on cankers, usually associated with older cankers. In *Eucalyptus* spp. is due to injury of the cambium, resulting in the formation of kino ducts (Conradie *et al*., 1990).

The results of the current pathogenicity screening study were used to select four isolates (F17, A17, G77 and E81) (Table 3.5) for further studies (Chapter 4).

Table 3.5 *Endothiella eucalypti* isolates selected for further studies.

Isolate	Collection Location	Pathogenicity rating
F17	Esperance	non-pathogenic
A17	Albany	weakly pathogenic
G77	Denmark	moderately pathogenic
E81	Manjimup	highly pathogenic

Temperature response of *Endothiella eucalypti in vitro*

Incubation temperature and collection location significantly ($p<0.0001$ and $p=0.004$, respectively) influenced mycelial growth (Table 3.6) and dry weight ($p<0.0001$ and $p=0.003$, respectively) of the *En. eucalypti* isolates *in vitro* (Table 3.7).

There was a high correlation between mycelial radial growth and dry weight at the four temperatures ($r=0.93$), therefore patterns in mycelial growth are discussed further. Overall, there was a significant ($p<0.0001$) difference in mycelial growth rate between the isolates (Table 3.8). Mycelial growth was significantly ($p\leq0.01$) greater at 25 °C compared to all other temperatures and significantly less ($p\leq0.01$) at 15 °C (Figure 3.6). Mycelial growth was visibly more dense at 30°C (Figure 3.7).

The mycelial growth rate of isolates (at each of the four temperatures studied) collected from Esperance was overall significantly ($p\leq0.01$) greater than the growth of isolates collected from the other five locations (Figure 3.6). There was a significant ($p<0.0001$) interaction between the isolate and temperature at each of the collection locations (data not shown).

Growth of individual isolates at the various temperatures could not be classified according to collection location. For example isolates D32 (Albany), E51 (Manjimup), E81(Manjimup) and G62 (Denmark) grew most rapidly at 25 °C, whilst isolate G38 (Denmark) had the smallest radial mycelial growth at this temperature. Further, there was no correlation ($r=0.013$) between growth rate at 25 ˚C and lesion extension (mm/week) (virulence). The colony morphology types (established in Chapter 2) did not generally relate to mycelial growth rate of *En. eucalypti* isolates at 25 °C. However, isolates B60, H29 and A17 which grouped into the Morphology type 2 were slow growers (36-42 mm/week) (Table 3.9).

Table 3.6 ANOVA of mycelial growth (mm/week) of *Endothiella eucalypti* incubated at 15, 20, 25 or 30 ℃ for one week. Significant values in bold font.

Effect	MS Effect	MS Error	F(df)	P
Location (1)	697.52	195.939	3.560 (5, 300)	**0.004**
Temperature (2)	37278.69	195.939	190.256 (3, 300)	**≤0.0001**
(1) x (2)	198.82	195.939	1.015 (15, 300)	0.439

Table 3.7 ANOVA of mycelial dry weight (mg/week) of *Endothiella eucalypti* incubated at 15, 20, 25 or 30 ℃ for one week. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Location (1)	753.41	204.839	3.678 (5, 299)	**0.003**
Temperature (2)	22771.01	204.839	111.165 (3, 299)	**≤0.0001**
(1) x (2)	219.73	204.839	1.073 (15, 299)	0.381

Table 3.8 ANOVA of mycelial growth (mm/week) of *Endothiella eucalypti* incubated at 15, 20, 25 or 30 ℃ for one week. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Isolate	1443.670	500.081	2.887 (26, 297)	**≤0.0001**

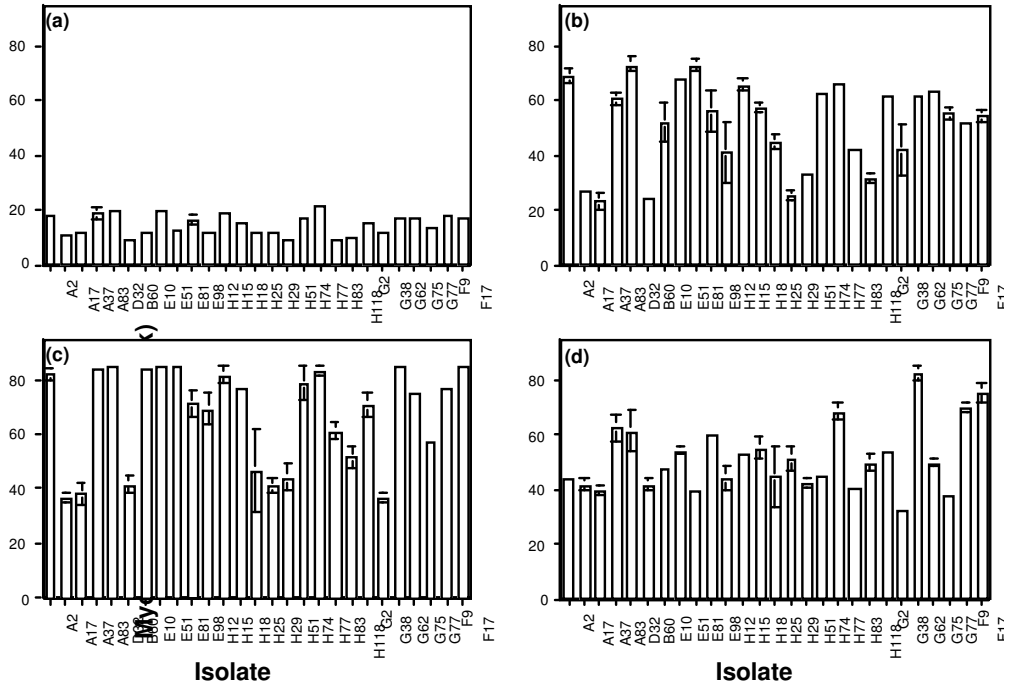


Figure 3.6 Average mycelial growth (mm/week) of *Endothiella eucalypti* isolates collected from Albany (A2, A17, A37, A83, D32), Manjimup (B60, E10, E51, E81, E98), Margaret River (H12, H15, H18, H25, H29), Bunbury (H51, H74, H77, H83, H118), Denmark (G2, G38, G62, G75, G77) and Esperance (F9, F17) incubated at (a) 15 ℃, (b) 20 ℃, (c) 25 ℃ and (d) 30 ℃. Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal.

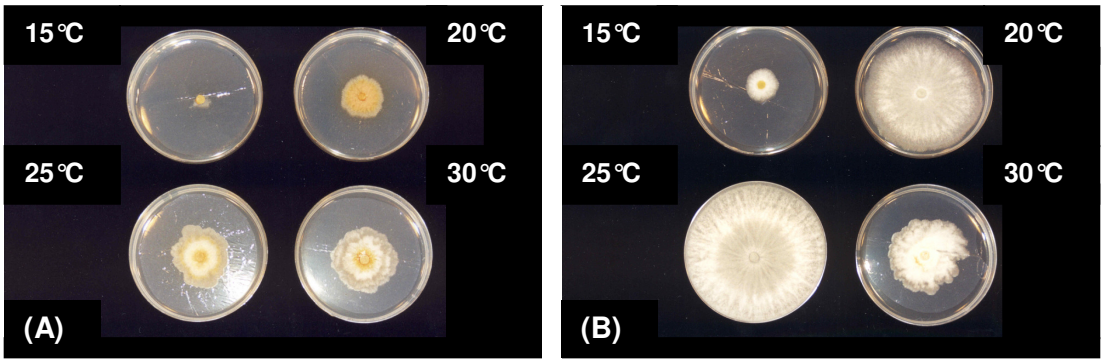


Figure 3.7 Mycelial growth of slow (A) and fast (B) growing *Endothiella eucalypti* isolates A17 and D32, respectively, incubated at 15, 20, 25 and 30 ℃ for one week.

Table 3.9 Growth (mm/week) at 25 ℃ and morphology type of *Endothiella eucalypti* isolates grown on half strength potato dextrose agar.

Endothiella eucalypti isolate	Growth at 25 ℃ (mm/week)	Morphology Type (from Chapter 2)
D32	85	5
G62		5
E51		2
E81		3
F17	84	4
E10	83.8	4
A83	83.6	6
H77	83	9
A2	82	1 and 3
H15	81.6	4
H74	78.3	1 and 4
H18	76.6	1 and 4
F9	76.3	6
G75	75.2	8
E98	71	5
G2	70.5	4
E69	69.5	3
H12		1 and 4
G77	57.5	4
H118	51.8	5
H25	46.6	1
H51	44.2	1
B60	41.6	2
H29	41.2	1 and 2
A17	36.8	2
G38	36.6	7

Pathogenicity of *Endothiella eucalypti* in excised *Eucalyptus globulus* stems at four temperatures

As temperature increased, lesion extension in excised stems of *E. globulus* also increased significantly (p=0.0001) (Table 3.10; Figure 3.8). Lesion extension (mm/week) was significantly (p<0.001, p=0.003 and p=0.02) greater at 30 °C than at 15, 20 and 25 °C, respectively. There was no (p=0.48) difference in extension of the lesions in stems incubated at 20 and 25 °C. Stem diameter had no (p=0.48) impact on the size of the lesions. There was no correlation (r=0.007) between the mycelial growth rate (colony diameter) of isolate E81 at 25 °C and lesion extension (mm/week) induced by this isolate at this temperature.

Table 3.10 ANOVA of lesion extension (mm/week) in excised stems of *Eucalyptus globulus* inoculated with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Temperature	2033.625	168.040	12.102 (3, 18)	**0.0001**

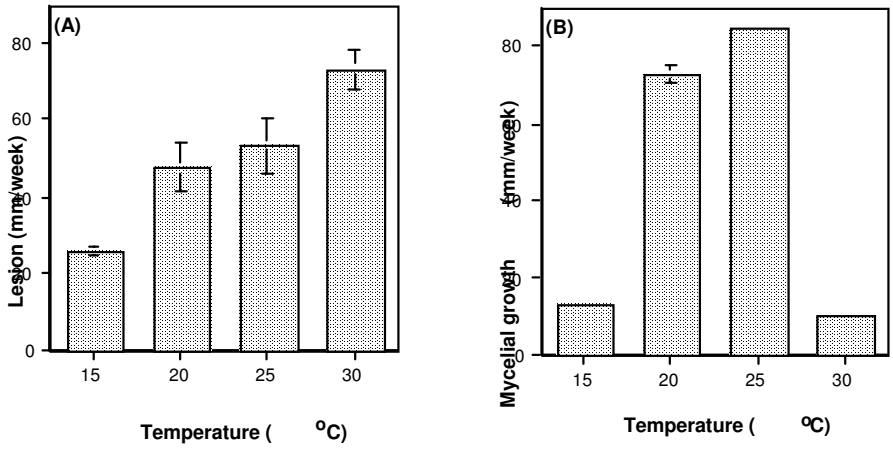


Figure 3.8 (A) Lesion extension (mm/week) in excised stems of *Eucalyptus globulus* inoculated with *Endothiella eucalypti* isolate E81 and incubated at 15, 20, 25 and 30 °C. **(B)** Average mycelial growth (mm/week) of *En. eucalypti* isolate E81 incubated at 15, 20, 25 and 30 °C for one week. Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal.

DISCUSSION

The *En. eucalypti* isolates collected from *E. globulus* plantations in southwestern Australia were highly variable in growth response to temperature and in pathogenicity. A high degree of variability in colony morphology within the WA population of *En. eucalypti* was previously noted in Chapter 2.

When inoculated under the bark the majority (96%) of the *En. eucalypti* isolates examined in the current study caused disease in stems of *E. globulus* saplings. Although pathogenicity studies of this pathogen on eucalypts have been conducted under a range of environmental conditions, some of the *En. eucalypti* isolates from WA produced larger lesions than these previous studies (Gryzenhout et al., 2003; Yuan and Mohammed, 2000; Shearer and Fairman, unpubl). For example, Gryzenhout and coworkers (2003) examined the pathogenicity of 15 *C. eucalypti* isolates in a two-year-old coppice stand of an *E. grandis* clone in South Africa and observed lesions which ranged in growth rate from 14.3 to 19.3 mm per week. Yuan and Mohammed (2000) inoculated one-year-old *E. globulus* and *E. nitens* saplings in an open-sided shade house in Hobart (Tasmania) with isolates of *C. eucalypti* and their *En. eucalypti* anamorph (of which two were from WA). One of the *En. eucalypti* isolates from WA produced larger lesions (growth rate 2.1 mm/week) in stems of *E. globulus* seedlings than those caused by its teleomorph (0.3 to 1.4 mm/week) (Yuan and Mohammed, 2000). These lesions were much smaller than those observed in the stems of one-year-old *E. globulus* saplings inoculated with *En. eucalypti* in the current study (12 and 30 mm/week). In addition, Shearer and Fairman (unpubl) inoculated the stems of two-year-old *E. globulus* with *C. eucalypti* in the glasshouse and recorded lesions ranging from 24.4 to 26.9 mm/week. Although all studies were conducted under different environmental conditions, the ability of *En. eucalypti* and its teleomorph to cause disease in eucalypts in the glasshouse or the field has been established.

There was a large variation in the rate of lesion extension between the *En. eucalypti* isolates inoculated into *E. globulus* in the current study. Previous studies have also reported a variation in pathogenicity between isolates of *C. eucalypti* and/or *En. eucalypti* in eucalypts (Gryzenhout *et al.*, 2003; Yuan and Mohammed, 2000; Old *et al.*, 1990). Yuan and Mohammed (2000) concluded that the fungal isolate and host species played important and unpredictable roles in determining the rate of disease development and suggested a possible host/ pathogen interaction. Further studies

examining the role of host genetics in determining the pathogenicity of *En. eucalypti* are addressed in Chapter 4.

The difference in the extent of lesion development between the glasshouse- and field-based pathogenicity studies is likely to be due to the different environmental conditions under which they were conducted. Previous studies have identified a strong interaction between the host genotype and the environment, which can affect the reproducibility of pathogenicity studies (Gryzenhout *et al.*, 2003; Old *et al.*, 1986). However, pathogenicity of *En. eucalypti* isolates in the plantation environment has been difficult to predict as studies by Yuan and Mohammed (2000) indicated that isolates collected from severely cankered eucalypts did not necessarily show the greatest pathogenicity in artificial inoculation trials. A comparison between *E. globulus* artificially inoculated with *En. eucalypti* in the field and glasshouse is discussed in Chapter 4.

Isolate F17 did not induce lesions in stems of *E. globulus* saplings after twenty-one days, however callus formed on the side of the stem opposite the inoculation point. Fungal isolates unable to induce symptoms of disease on a given plants species are considered non-pathogenic on that species (Olivain and Alabouvette, 1997). Therefore, *En. eucalypti* isolate F17 may be regarded as non-pathogenic to *E. globulus*. A non-pathogenic isolate can be used to protect an incompatible plant against its specific pathogen (Olivain and Alabouvette, 1997). Previous studies on *Vigna sinensis* (cowpea) and *Lycopersicon esculentum* (tomato) plants have shown that non-pathogenic isolates of *Uromyces appendiculatus* and *Fusarium oxysporum*, respectively, are effective in inducing a host defence response without causing disease, thereby 'priming' the host for future pathogen attack (Fink *et al.*, 1991; Olivain and Alabouvette, 1997). The host is then able to quickly and effectively slow and/or stop future pathogen ingress (Fink *et al.*, 1991; Olivain and Alabouvette, 1997). If, through further studies (Chapter 4), this isolate does prove to be non-pathogenic it could be very useful in inducing the host defence responses of *E. globulus* and thus protecting it against future challenge by a more aggressive isolate or pathogen.

Measurement of lesion extension gave an accurate indication of *En. eucalypti* colonisation of the *E. globulus* stems and therefore its relative pathogenicity to the host. In contrast, pathogens such as *Phytophthora cinnamomi* can extensively colonise the host without inducing host wound responses or causing visible lesion development (O'Gara *et al*., 1997; Huberli *et al.*, 2000; Huberli *et al.*, 2002). Therefore, unlike

En. eucalypti, it is impossible to determine the damage caused by *P. cinnamomi* to the host by visual means alone (Huberli *et al*., 2000).

Orange pycnidia, indicating the presence of *En. eucalypti,* were often present on the stems of plantation-grown *E. globulus* with or without an accompanying lesion (Chapter 2). In the current study, lesions were frequently accompanied by pycnidia, regardless of the pathogenicity of the isolate. However, it appears that *En. eucalypti* can survive on healthy stems until conditions are optimal for invasion of the host. This also facilitates the build-up of the pathogen within the plantation environment over time. Further studies investigating the infection process of *En. eucalypti* are covered in Chapter 5.

Sapwood invasion by canker fungi often results in discolouration of the wood at the site of pathogen penetration/invasion, resulting in reduced water movement through the colonised wood and ultimately wilting of leaves above the site of the canker (Old and Davison, 2000). Wilting was observed in plants inoculated with the more pathogenic isolates (E81, H74, H12). Isolate E81, which induced the largest lesions in the current study, also girdled the *E. globulus* saplings, resulting in the drying of leaves and eventual death of the top of the plant. This indicates that more aggressive *En. eucalypti* isolates are capable of causing serious damage to *E. globulus* and may cause death if the lesion girdles the stem.

The growth rate of the *En. eucalypti* isolates *in vitro,* at temperatures ranging from 15 to 30 °C, did not group the isolates according to the geographical region from which the isolates were collected. Generally, an isolate which grew at a faster rate at 25 ˚C also grew at a faster rate (compared to the majority of the isolates) at 15, 20 and 30 ˚C. Exceptions to this trend were noted for isolates E81, A2 and H74, for which growth was slowed at 30 °C. In addition, isolate B60 was a slow grower at all four temperatures. The lack of relationship between growth rate and collection location was not unexpected as the temperature study conducted by Venter *et al*. (1999) was later found to have grouped the isolates according to species (Venter *et al*., 2002). The *End. gyrosa* isolates from North America were separated by *in vitro* growth from the *C. eucalypti* isolates from South Africa and Australia. Therefore, the extent of geographical variation within southwestern Australia was not great enough to separate the *En. eucalypti* isolates into groups according to collection location over the range of

temperatures studied. However, use of molecular techniques (such as those used in Chapter 6) may be more useful in grouping *En. eucalypti* isolates.

The level of disease expression depends on the aggressiveness of the pathogen and the rate at which the host responds (Fraser and Davison, 1985). The potential of *En. eucalypti* to cause disease in excised *E. globulus* stems was greatest at the highest temperature studied (30 °C). In contrast, at 30 ˚C the growth rate of these isolates *in vitro* dropped by half and the mycelial growth form of all *En. eucalypti* isolates became visibly more compact. The role of humidity in facilitating pathogen invasion and spread within host stems needs to be addressed, as high humidity may allow survival of the pathogen outside its optimal temperature range.

Drought has been identified as a factor which reduces the resistance of eucalypts to canker fungi (Old and Davison, 2000; Old *et al.*, 1990; Swart *et al.*, 1992; Schoeneweiss, 1981). However, studies by Paap (2001) indicated that extension of *Endothiella* in excised *Co. calophylla* stems was limited in plants maintained below field capacity. In addition, cankers caused by *P. cinnamomi* were more extensive in hydrated stems of *E. marginata* than when bark water content was low (Tippett *et al.*, 1987). Further studies of both excised and intact eucalypt stems are required to determine the role of temperature, humidity and host water content on host susceptibility to *En. eucalypti*.

In conclusion, there was a high degree of variability in growth rate, response to temperature and pathogenicity to *E. globulus* within the *En. eucalypti* isolates studied. As reported here and in previous studies (Yuan and Mohammed, 2000), the variation between isolates of *En. eucalypti* cannot be linked with location of isolate collection or culture age. Therefore, in addition to screening the pathogenicity of *En. eucalypti* isolates to various *E. globulus* provenances, as covered in Chapter 4, further studies on population genetics of this pathogen are required (Chapter 6). In addition, information on the patterns/mechanisms of pathogen dispersal are required in order to assess the potential impact of *En. eucalypti* on WA *E. globulus* plantations in the future.

Chapter 4

Susceptibility of *Eucalyptus globulus* provenances to *Endothiella eucalypti* in the glasshouse and in the field

INTRODUCTION

Variation in the pathogenicity of *Endothiella eucalypti* was observed when artificially inoculated into stems of one-year-old *Eucalyptus globulus* under controlled conditions (Chapter 3). However, in plantations Old and Davison (2000) concluded that host susceptibility to a pathogen resulted from either: (i) the susceptibility of the host species or provenance, (ii) the ability of a pathogen to cause disease, or (iii) the build up of inoculum on alternate hosts. Therefore, pathogenicity ratings established in artificial inoculation trials under controlled conditions may not correlate with the level of disease expressed in the field (Yuan and Mohammed, 2000) as the host may be predisposed to infection by environmentally induced stress (discussed in Chapters 7 and 8).

The cause of a severe canker outbreak in plantation-grown *E. nitens* in Tasmania was attributed to infection by *Cryphonectria eucalypti,* with some provenances more affected than others (Wardlaw, 1999). Artificial inoculation studies investigating the susceptibility of different *Eucalyptus* species (Yuan and Mohammed, 2000; Old *et al.*, 1986; Shearer and Fairman, unpubl.) and clones to *C. eucalypti* (Gryzenhout *et al.*, 2003), showed a variation in susceptibility between species and within clones of the same species. In addition, one of the field-based studies concluded that although host susceptibility played a role in disease development, this process was also strongly influenced by environmental factors (Gryzenhout *et al.*, 2003).

Cryphonectria cubensis causes severe stem cankers resulting in significant losses in eucalypt plantations in tropical and subtropical areas of the world (van Heerden and Wingfield, 2002; van Zyl and Wingfield, 1999; Campinhos and Ikemori, 1983). Currently, use of resistant or less susceptible eucalypt species is the only means of reducing losses from damage by *Cryphonectria* among eucalypt species in Brazil and South Africa (van Heerden and Wingfield, 2002; Conradie *et al.*, 1990). Vegetative propagation of *Eucalyptus* clones is widely practised to improve timber quality and yield, however clonal forestry may increase the threat of disease outbreaks (Gryzenhout *et al.*, 2003; Heather and Griffin, 1984). In the absence of a clonal forestry program for *E. globulus* selection in Australia, breeding in seed orchards for more superior seed

sources (provenances/families), for faster growing varieties or those with a greater tolerance to low moisture conditions, is currently the only method of determining seed for future plantings. As *En. eucalypti* is widespread in *E. globulus* plantations in southwestern Australia, resistance to this pathogen and other fungal diseases may also need to be considered when selecting seed sources for future plantations in WA. Therefore, the aims of the study reported in this chapter were to investigate the susceptibility of different *E. globulus* provenances, currently being planted in southwestern Australia, to *En. eucalypti* under glasshouse and field conditions and to determine the validity of screening provenances for resistance to *En. eucalypti* in the glasshouse.

MATERIALS AND METHODS

Two experiments were undertaken. The first experiment examined the pathogenicity of four *En. eucalypti* isolates on seven *E. globulus* provenances under glasshouse conditions. The second experiment, conducted at two plantations in the Albany region, examined the pathogenicity of 29 *En. eucalypti* isolates on three *E. globulus* provenances. Two of these provenances (A and W) were included in the glasshouse trial.

Pathogenicity of four *Endothiella eucalypti* isolates to seven *Eucalyptus globulus* provenances under glasshouse conditions

Experimental design

The susceptibility of seven *E. globulus* provenances to four *En. eucalypti* isolates was investigated in a glasshouse. The *E. globulus* plants were underbark inoculated on the main stem with one of four *En. eucalypti* isolates (E81, F17, G77 and A17) tested in Chapter 3 (Table 3.5). The control treatments consisted of sterile non-colonised Miracloth® discs. Five replicate *E. globulus* seedlings were inoculated for each treatment with the plants arranged in a randomised complete block design (Steel and Torrie, 1986). Four weeks after inoculation, the stems were harvested and lesion extension and colonisation were measured.

Biological materials

Six-month-old *E. globulus* seedlings, supplied by ITC Ltd. (Albany, WA), were grown in 150 mm free-draining plastic pots containing potting mix (Chapter 3) under glasshouse conditions. Provenances B and W were open pollinated lines from a seed orchard in mainland Australia. The remaining provenances were as follows; FI

(Flinders Island), KI (King Island), G (Greeveston), A (Amcor, Victoria) and O (Otway). Plants were fertilized (5 g per pot of each) with six month slow-release fertiliser (Scotts Osmocote Plus, Scotts Europe BV, Heerlen, The Netherlands) and isobutylidene diurea (IBDU - Mitsubishi Chemical Corp, Chiyodaku, Tokyo Japan). The experiment was conducted in an evaporatively cooled glasshouse (20 - 27 ˚C min-max) in late summer (February) and the plants were watered overhead twice a day for 20 minutes. At the time of the experiment the plants were two-years-old, approximately 1 m in height, with an average stem diameter of 10 mm measured 200 mm from the base.

Prior to use, the four *En. eucalypti* isolates A17 (Albany), E81 (Manjimup), F17 (Esperance) G77 (Denmark), selected from the 29 isolates in Chapter 3 (Table 3.5), were passed through healthy *E. globulus* stems (Chapter 3). Once re-isolated, they were maintained on 1/2PDA plates at 24 ˚C under continuous near blue light. Actively growing cultures of each isolate were subcultured onto plates containing sterile Miracloth® discs (Chapter 3). Sterile non-colonised discs were used as controls.

Inoculation and harvest

The main stem of each plant was underbark inoculated (approximately 200 mm up from the base of the stem) with an *En. eucalypti* colonised disc or sterile non-colonised disc (control treatment) (as outlined in Chapter 3).

At harvest, four weeks after inoculation, lesion extension and pathogen colonisation of the stem were measured (Chapter 3). Stem segments (10 mm in length) were plated onto 1/2PDA+S plates and fungal growth monitored from each segment over seven days. Colonisation beyond the lesion was then determined as described in Chapter 3. The inoculum discs were recovered from each of the stems and plated onto 1/2PDA+S in order to check the viability of the inoculum.

Pathogenicity of twenty-nine *Endothiella eucalypti* isolates to three *Eucalyptus globulus* provenances under field conditions

Experimental design

The pathogenicity of 29 *En. eucalypti* isolates and one *C. cubensis* isolate (as a comparison) was examined in 18-month-old *E. globulus* saplings at two ITC Ltd. plantations (Marri Downs and Komodo) in the Albany region (Table 4.1; Figure 2.1). At each plantation, three *E. globulus* provenances (C, A and W) were planted in rows within blocks, which were located in close proximity to each other. A side branch from each tree (average diameter of 6 mm) approximately 1 m up from the base of the main stem was underbark inoculated. Branches were harvested 18 weeks after inoculation. At each location, five replicate trees from each provenance were inoculated (one replicate per row of trees) (Appendix Table 4.1). The minimum number of replicates required to provide reliable results in the current field trial was established using a Power Analysis (SPSS for Windows 10.0.5; SPSS Inc. 1989-1999) on previous findings in the field (Jackson, 2000).

Site descriptions and Provenance details

Marri Downs (889 ha) plantation, approximately 40 km west of Mount Barker, is situated on dark brown fertile loamy sands, over gravelly sandy/loams, over moderately structured clays (Figure 2.1, plantation number 27). This region has an average annual rainfall of 735 mm. Komodo (106.6 ha), located 55 km northeast of Albany, is situated on duplex soils with an anaerobic subsoil. The area has an average annual rainfall of 640 mm (Figure 2.1, plantation number 28). The plantations were established by ITC in 2001, planted approximately 2 m apart within rows and 2.5 m between rows (Figure 4.1). The *E. globulus* provenance, A originated from an open pollinated seed orchard located in Gippsland, C from the central west area of Flinders Island and W from an open pollinated seed orchard located at Mt Gambier.

Fungal isolates, selection of trees and inoculation

The pathogenicity of the 29 *En. eucalypti* isolates used in Chapter 3 and a single isolate of *C. cubensis* isolated from diseased *E. marginata* (DCLM) in southwestern Australia was examined (Table 4.1). The *C. cubensis* isolate was included as a comparison. Isolates were maintained on 1/2PDA plates at 24˚C under continuous near blue light. Inoculum and non-colonised discs (control treatment) were prepared as described in previous experiment.

Figure 4.1
The two-year-old Marri Downs plantation located west of Mount Barker.

Within in the rows (where possible), visually healthy trees, with no obvious nutrient deficiency symptoms (Dell *et al.*, 2001) and no adult foliage, were randomly selected for inoculation. Branches were underbark inoculated (Chapter 3) approximately one third of the distance along the branch from the main trunk (Figure 4.2). A colonised disc (or sterile control disc) was inserted into a shallow incision, the wounded stem was wrapped in Parafilm (American National Can™), ducting tape and flagging tapes to avoid fungal desiccation and light penetration. At the time of inoculation in November 2000, the trees were 18-months-old and approximately 2 m in height.

Figure 4.2
Insertion of a Miracloth® disc, colonised with *Endothiella eucalypti* into the branch of a two-year-old *Eucalyptus globulus* tree.

Harvest

Eighteen weeks after inoculation (at which stage lesions were clearly observed on branches), the branches were harvested, placed in black plastic bags and immediately returned to the laboratory where lesion extension and stem diameters were measured. Three stems from each treatment (a total of 930 branches) were plated onto 1/PDA+S (as described in Chapter 3) to determine pathogen colonisation of the stem.

Statistical analysis

Lesion extension (mm/week), colonisation beyond the lesion (mm/week) and stem diameter were analysed separately by ANOVA using Statistica Version 4.1 (StatSoft® Inc., OK, USA, 1991-1994). Data were assessed for, and met the requirements of, homogeneity, variation of the mean from the variance and fit to a normal distribution. In the glasshouse trial, means were compared by LSD ($p \leq 0.05$) and are presented with standard error of the mean. In the field trial, stem diameter was assessed as a covariate, means were compared by LSD ($p \leq 0.01$) and presented with standard error of the mean.

Table 4.1 Location and plantation of initial isolate collection for each of the selected *Endothiella eucalypti* isolates (A-H) and the *Cryphonectria cubensis* (C) isolate.

Isolate Number	Isolate Code	Collection location	Plantation Number (Fig 2.1)
1	A2	Albany	22
2	A17		22
3	A83		21
4	D42		20
5	D32		19
6	B60	Manjimup	9
7	E10		7
8	E51		7
9	E81		11
10	E98		8
11	E69		11
12	F9	Esperance	25
13	F17		25
14	G2	Denmark	14
15	G38		13
16	G62		17
17	G75		16
18	G77		15
19	H12	Margaret River	6
20	H15		6
21	H18		6
22	H25		5
23	H29		5
24	H46		4
25	H51	Bunbury	2
26	H57		2
27	H74		1
28	H77		1
29	H118		3
30	CRY B (DCE 384)	*Eucalyptus marginata* in WA	NA
31	CONTROL	NA	NA

RESULTS

Pathogenicity of four *Endothiella eucalypti* isolates to seven *Eucalyptus globulus* provenances under glasshouse conditions

The four *En. eucalypti* isolates formed lesions in all of the *E. globulus* provenances. As there was a high correlation (r = 0.81) between lesion extension and total colonisation (Figure 4.3), pathogenicity is considered only in relation to lesion extension.

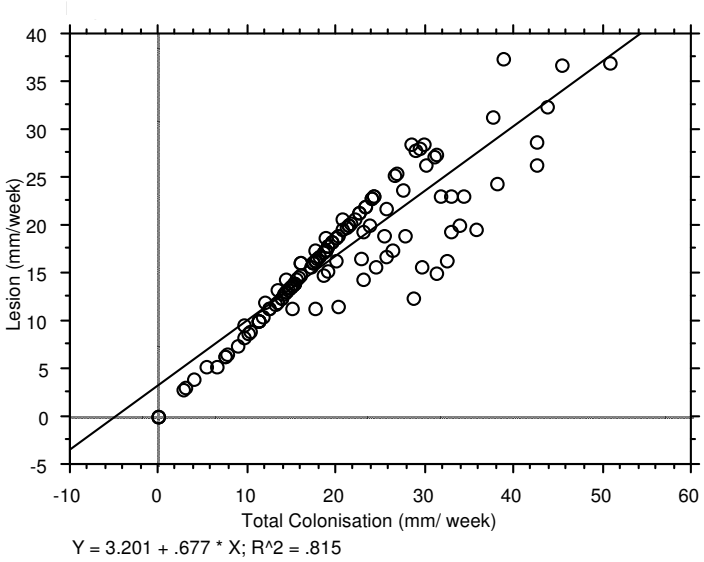


Figure 4.3 Correlation between lesion development (mm/week) and total colonisation (mm/week) in the stems of two-year-old *Eucalyptus globulus* provenances inoculated with one of four *Endothiella eucalypti* isolates A17, E81, F17 and G77.

There was a significant (p=0.004) interaction between *En. eucalypti* isolate and *E. globulus* provenance (Figure 4.4). Provenance had a significant (p<0.0001) effect on lesion extension (Table 4.2). Provenance significantly (p=0.01) influenced stem diameter, provenance B had the largest stem diameter (Figure 4.6; Table 4.3). There was no relationship between stem diameter and lesion length.

There was a significant (p<0.0001) difference in lesion extension between isolates across all provenances (Table 4.2). Isolate E81 caused significantly (p<0.0001) larger lesions than the other isolates over all provenances (Figure 4.5 and 4.7). In comparison, isolate A17 induced the smallest lesions in provenance KI, G, A, and O, however, it caused the second largest lesions in provenance FI, W and B (Figure 4.5 and 4.7). Although isolate E81 was the most pathogenic isolate over all the provenances there was no clear pattern in pathogenicity for the other three isolates.

Provenance A was the only provenance in which inoculation with any of the four isolates did not completely girdle stems. Isolates G77 and A17 were less likely to girdle provenance KI, with 80% and 60% of inoculated stems girdled, respectively.

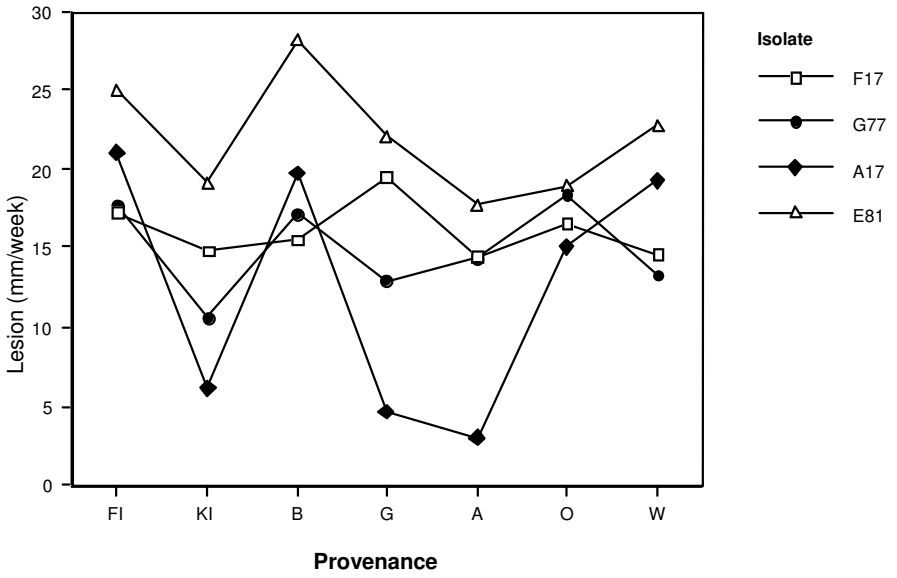


Figure 4.4 Two-way interaction plot (f (18,104)=2.32: p<0.004) for lesion (mm/week) in stems of seven *Eucalyptus globulus* provenances (FI, KI, B, G, A, O, W) inoculated with four *Endothiella eucalypti* isolates A17, E81, F17 and G77.

Table 4.2 ANOVA of lesion extension (mm/week) in stems of *Eucalyptus globulus* seedlings following inoculation with *Endothiella eucalypti*. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Provenance (1)	193.362	28.987	6.670 (6, 104)	**≤0.0001**
Isolate (2)	526.977	28.987	18.180 (3, 104)	**≤0.0001**
1 x 2	67.379	28.987	2.324 (18, 104)	**0.004**

Table 4.3 ANOVA of stem diameter (mm) of stems of *Eucalyptus globulus* seedlings. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Provenance (1)	4.888	1.647	2.968 (6, 104)	**0.010**
Isolate (2)	0.708	1.647	0.430 (3, 104)	0.732
1 x 2	0.970	1.647	0.589 (18, 104)	0.900

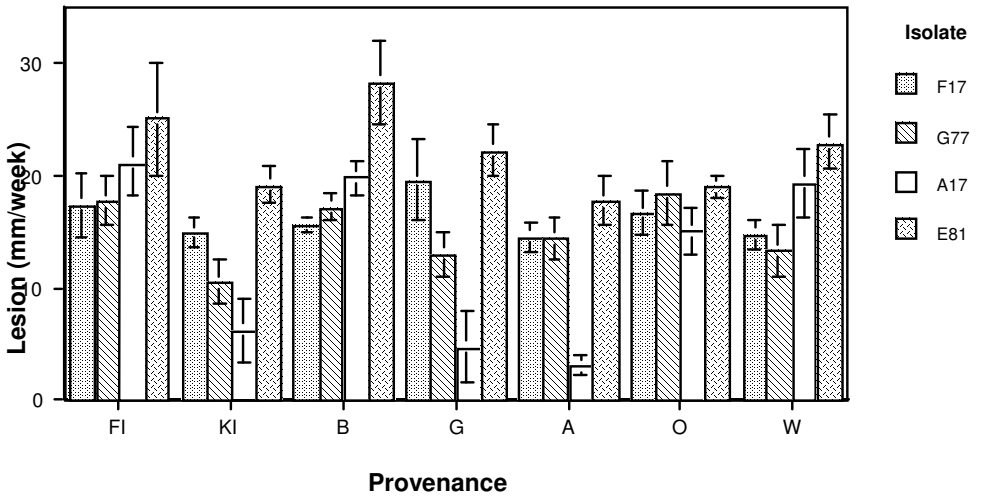


Figure 4.5 Lesion extension (mm/week) in the stems of seven *Eucalyptus globulus* provenances (FI, KI, B, G, A, O and W) inoculated with *Endothiella eucalypti* isolates F17, A17, G77 and E81 in the glasshouse.

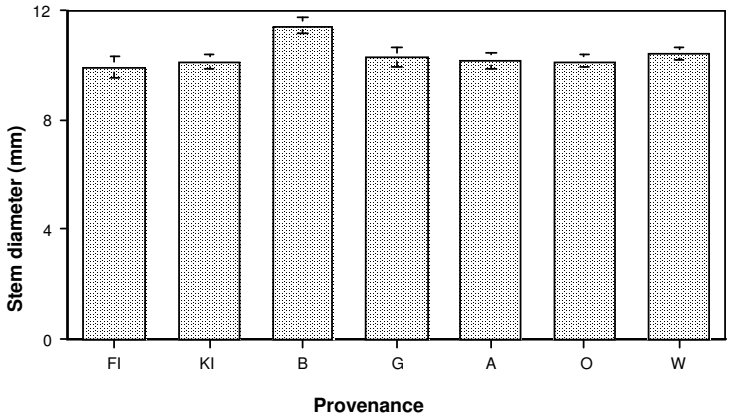


Figure 4.6 Average stem diameters (mm) of the *Eucalyptus globulus* provenances (FI, KI, B, G, A, O and W) measured 200 mm from the base of the stem. Bars indicate standard errors of the mean.

Figure 4.7 Examples of lesion development in stems of *Eucalyptus globulus* of provenance G inoculated with highly pathogenic *Endothiella eucalypti* isolate E81 (left) and weakly pathogenic isolate A17 (right). Arrows indicate ends of lesions.

Pathogenicity of twenty-nine *Endothiella eucalypti* isolates to three *Eucalyptus globulus* provenances under field conditions

Observations

The *E. globulus* at the Marri Downs plantation were visually more healthy and generally taller than those at Komodo. At Marri Downs, *E. globulus* showed minimal signs of Mycosphaerella Leaf Disease (MLD) or insect attack compared to Komodo where the majority of the trees were affected by *Phylacteophaga froggatti* (leaf blister saw fly). MLD was also more evident at Komodo than Marri Downs.

Lesion development

Endothiella eucalypti caused significantly ($p<0.0001$) larger lesions on *E. globulus* at Marri Downs (2.94 ±0.04 mm/week) compared to Komodo (2.60 ± 0.04 mm/week) (Tables 4.4 and 4.5). There was a significant ($p=0.01$) interaction between plantation and *E. globulus* provenance (Figure 4.8, Table 4.4). Provenance A had the largest lesions (2.84 ± 0.04 mm/week), followed by provenance C (2.76 ± 0.05 mm/week) and the smallest were in provenance W (2.73 ± 0.05 mm). However, the difference between provenances was not significant ($p=0.437$) (Table 4.4). Provenance W developed the

largest and smallest lesions when inoculated at Marri Downs (2.96 ± 0.08 mm) and Komodo (2.52 ± 0.05 mm) (Table 4.5; Figure 4.8), respectively.

There was a highly significant ($p<0.0001$) difference in lesion extension between the *En. eucalypti* isolates (Table 4.4). Overall, isolate H29 (Margaret River) formed the largest lesions (3.10 ± 0.22 mm/week) in *E. globulus* branches followed by isolate H12 (Margaret River) (3.06 ± 0.14 mm/week). All *En. eucalypti* isolates induced significantly ($p<0.0001$) larger lesions than the *C. cubensis* isolate (1.58 ± 0.13 mm/week) in *E. globulus*. *Endothiella eucalypti* isolates G38 (Denmark) (2.13 ± 0.12 mm/week) and F17 (Esperance) (2.27 ± 0.08 mm) formed the smallest lesions over both locations and all provenances (Figure 4.9).

Table 4.4 ANOVA of lesion extension (mm/week) in stems of three 18-month-old *Eucalyptus globulus* provenances (A, C and W) at Marri Downs and Komodo plantations inoculated with 29 *Endothiella eucalypti* isolates and one *Cryphonectria cubensis* isolate. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Location (1)	17.230	0.371	46.420 (1, 501)	**≤0.0001**
Provenance (2)	0.308	0.371	0.829 (2, 501)	0.437
Isolate (3)	2.284	0.371	6.155 (29, 501)	**≤0.0001**
1 x 2	1.693	0.371	4.560 (2, 501)	**0.010**
1 x 3	0.347	0.371	0.935 (29, 501)	0.565
2 x 3	0.315	0.371	0.848 (58, 501)	0.779
1 x 2 x 3	0.388	0.371	1.046 (58, 501)	0.389

Table 4.5 Mean lesion extension (±SE) in stems of three 18-month-old *Eucalyptus globulus* provenances (A, C and W) at Marri Downs and Komodo plantations inoculated with 29 *Endothiella eucalypti* isolates and *Cryphonectria cubensis* isolate.

Location	**Provenance**	**Mean lesion (mm/week)**	**± SE**
Marri Downs	A	2.95	0.061
	C	2.89	0.074
	W	2.96	0.076
Komodo	A	2.69	0.064
	C	2.61	0.069
	W	2.52	0.054

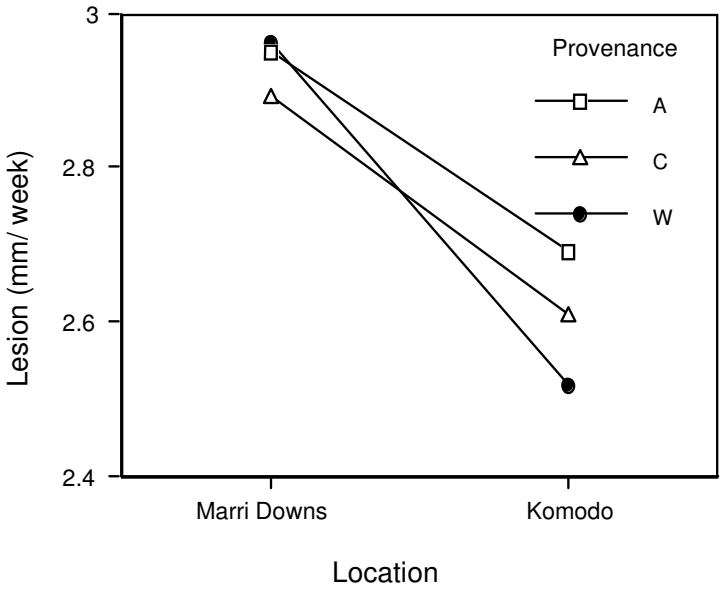


Figure 4.8 Two-way Interaction plot (f(2, 502)=4.53; p<0.0112) for lesion extension (mm/week) in stems of 18-month old *Eucalyptus globulus* of three provenances (A, C and W) inoculated with *Endothiella eucalypti* at two field locations (Marri Downs and Komodo).

Figure 4.9 Average lesion (mm/week) (±SE) in the stems of three 18-month-old *Eucalyptus globulus* provenances (A, C and W) caused by 29 *Endothiella eucalypti* isolates and one *Cryphonectria cubensis* isolate at the Marri Downs and Komodo plantations. Isolates 19 and 20 at Marri Downs and 4, 6, 8, 11 and 19 at Komodo indicate missing data rather than no lesion development. Bars indicate the standard error of the mean.

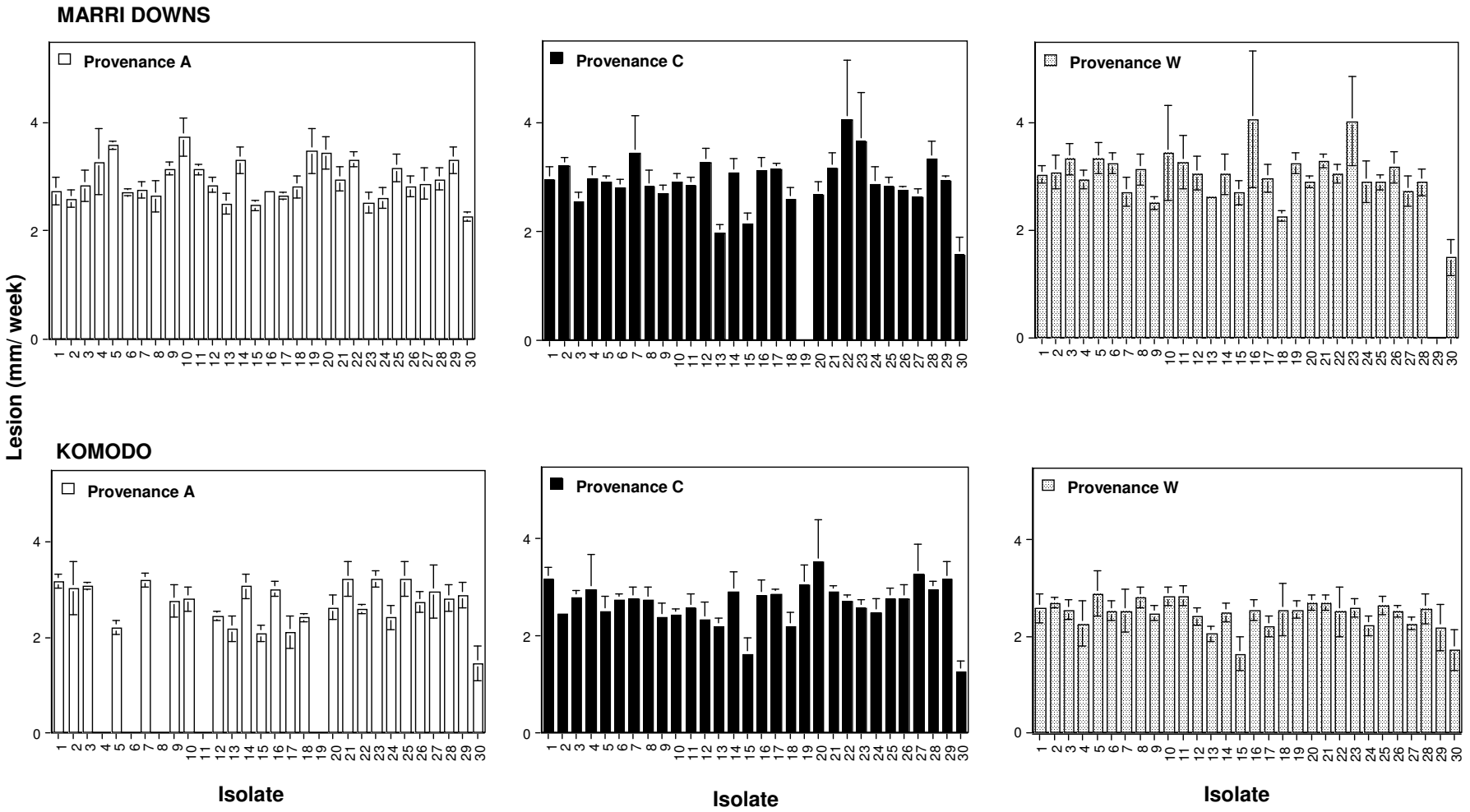


Figure 4.9 Average lesion extension (mm/week) (±SE) in the stems of three 18-month-old *Eucalyptus globulus* provenances (A, C and W) caused by 29 *Endothiella eucalypti* isolates and one *Cryphonectria cubensis* isolate at the Marri Downs and Komodo plantations. Isolates 19 and 20 at Marri Downs and 4, 6, 8, 11 and 19 at Komodo indicate missing data rather than no lesion development. Bars indicate the standard error of the mean. Where bars are not visible. standard errors are minimal.

DISCUSSION

Endothiella eucalypti produced lesions in all *E. globulus* provenances in the glasshouse and the field. The *E. globulus* provenances exhibited various levels of resistance to each of the *En. eucalypti* isolates. Although some isolates were consistently more pathogenic to all provenances, the plantation location significantly influenced provenance susceptibility to *En. eucalypti*.

Isolate E81 (from Manjimup) was consistently the most pathogenic isolate over all provenances under glasshouse conditions, in the current study and in the previous glasshouse trial (Chapter 3). In comparison, isolate F17 (from Esperance), which was non-pathogenic in the previous glasshouse trial (Chapter 3), caused the second largest lesions overall in the current glasshouse study. The variation in the extent of disease caused by isolate F17 may be attributed to the time of the year at which the experiments were conducted, with the experiments conducted in late spring (Chapter 3) and late summer (current study). However, both glasshouse trials were conducted in the same glasshouse under temperature controlled conditions. In comparison, under field conditions, isolate F17 consistently produced some of the smallest lesions across the plantations and provenances. Inoculation of *E. globulus* with isolate E81 under field conditions resulted in moderate lesion development overall compared with the other *En. eucalypti* isolates. In contrast to the glasshouse trials, this isolate did not prove to be the most pathogenic isolate, in any provenance, under field conditions.

There was no correlation in relative pathogenicity of isolates to *E. globulus* between the glasshouse and the field, with lesion extension for each isolate varying according to host provenance and plantation location. Although there was a significant interaction between provenance and isolate in the glasshouse, the results showed that provenance A was most resistant to three of the four fungal isolates and provenance K1 was the most resistant to one isolate (G77) and the second most resistant to the other three isolates. It may be concluded that provenances A and K1 were more resistant and provenances F1, B, O and W more susceptible under glasshouse conditions. In the field the relative resistance of provenances A and W was not evident. This may be attributed to environmental conditions in the glasshouse compared to the field. The results of the current studies indicate that determination of relative provenance resistance to *En. eucalypti* requires investigations which use a large number of isolates, on a range of provenances, under a range of field conditions. In addition, investigations which use a

less invasive method of inoculation may also aid in determining host susceptibility (Chapter 5).

Lesions caused by *En. eucalypti* generally extended more rapidly in the glasshouse than in the field. This may be a result of the more controlled temperature conditions, high humidity and adequate water supply in the glasshouse which created ideal conditions for pathogen growth and host colonisation. Under these conditions, the variation in the susceptibility of *E. globulus* provenances to *En. eucalypti* was marked. In comparison, under field conditions the variation in provenance susceptibility was smaller than that observed in the glasshouse. It appears that greater lesion development, as a result of inoculation with *En. eucalypti,* occurs under high stem moisture conditions, although additional studies are required to confirm this observation. Research into the responses of the pathogen to a range of host stem moisture conditions may aid in explaining variations in provenance susceptibility.

The severity of the disease caused by *En. eucalypti* differed depending on the geographical location of the plantation. Previous studies on *Eucalyptus* clones (*E. grandis, E. grandis x camaldulensis* and *E. grandis x urophylla*) in South Africa reported a variation in severity of disease caused by *C. cubensis* according to the location of the trial (van Heerden and Wingfield, 2002). In the current trial, the Komodo plantation was visually less healthy than Marri Downs, with leaf damage caused by insects and foliar disease (MLD) and generally smaller trees. Komodo receives a lower average annual rainfall of 640 mm compared to 735 mm received at Marri Downs. It may have been expected that a healthier, less stressed *E. globulus* provenance would be less affected by *En. eucalypti* infection than those stressed by nutrient deficiencies, defoliation (by insects or disease) or water deficit. However, this was not the case in the current study, as the *E. globulus* provenances at Komodo had smaller lesions than those at the Marri Downs plantation. Although water resource availability was not assessed at each of the plantations in the current trial, it is hypothesised that variations in stem moisture may explain the different responses of the *E. globulus* provenances at the two plantations as well as in the variation between the glasshouse and field trials. Alternatively, the reduction in foliage caused by insects and disease (MLD) at the Komodo plantation may have induced the host defence responses, thereby reducing the susceptibility of trees to inoculation with *En. eucalypti*. Induced chemical defense, in association with compensatory photosynthesis, has previously been linked to herbivore damage (Tiffin, 2000). However, without information on the

environmental conditions at the two sites, it is unclear whether the trees at the Komodo plantation were more stressed than those at Marri Downs and as a result were more affected by insects and disease, or whether the Komodo plantation had a higher level of insects and fungal spores in its vicinity which resulted in a highly stressed host at that site. Further physiological and biochemical studies are required in order to determine the role of environmental conditions, insect attack and foliar disease on the host defences of *E. globulus* when challenged by *En. eucalypti*.

In the current field study, the *C. cubensis* isolate consistently caused the smallest lesions of all the isolates, regardless of *E. globulus* provenance or plantation location. As *C. cubensis* is generally regarded as being more pathogenic than *En. eucalypti,* the results of the current study were unexpected. *Cryphonectria cubensis* is considered a serious stem pathogen to eucalypts in the tropical and subtropical regions of the world (Wingfield *et al.*, 2001; van Heerden and Wingfield, 2001; Old and Davison, 2000; Davison, 1995; Davison and Coates, 1991), favours disease expression in humid, moist conditions with temperatures above 23 °C (Conradie *et al.*, 1990; Swart *et al.*, 1992) and has been found in limited extent in WA associated with root cankers and a crown canker of *E. marginata* (Davison and Coates, 1991). The unexpectedly low pathogenicity observed for *C. cubensis* may be due to factors such as: (i) the low pathogenicity of the isolate used in the current trial; (ii) the environmental conditions within the *E. globulus* plantations in southwestern Australia were not conducive to *C. cubensis* disease expression; (iii) the isolate had been in culture for many years prior to its use and although it was passaged through a *E. globulus* host prior to its use in the current study, it is possible that long-term storage in culture effected its pathogenicity; and (iv) that the *C. cubensis* isolate was originally isolated from *E. marginata* and is not as pathogenic to *E. globulus*. Following the isolation of *C. cubensis* in WA, it was considered a possible disease risk if it was to move into *E. globulus* plantations (Davison, 1995). However, the results of the current study indicate that, perhaps due to environmental conditions, this fungus does not appear to pose an immediate disease threat in the areas studied. Further, *C. cubensis* was not isolated during the recent survey of canker fungi present in plantations in southwestern Australia (Chapter 2). However, monitoring the distribution of *C. cubensis* in southwestern Australia and its presence in *E. globulus* plantations in the future would be wise.

The susceptibility of eucalypts to invasion by canker fungi has been associated with the bark characteristics of the host. A survey of stem cankers associated with *C. eucalypti*

in a 14-year-old mixed provenance *Eucalyptus nitens* plantation in Tasmania suggested that trees with rougher bark were more likely to be associated with canker damage than smoother barked trees (Wardlaw, 1999). *Cryphonectria eucalypti* was isolated from fine longitudinal cracks (fissures) in new bark of rough-barked trees, but was not present in cracks on smooth-barked trees. It is suggested that the rough bark provides an infection court which promotes infection under ideal environmental conditions, thus increasing the susceptibility of the host to infection by *C. eucalypti*. However, the pathogen was not observed in similar aged rough-barked trees in provenance trials of *E. nitens* in other parts of Tasmania. Pathogenicity studies with isolates of *C. eucalypti* collected from this epidemic site showed that when the pathogen was wound inoculated into smooth- and rough-barked *E. nitens,* smooth-barked trees were more susceptible. However, when spores or mycelium of *C. eucalypti* were placed on the surface of intact bark, infections were only observed in rough bark trees. Although isolates of *C. eucalypti* were responsible for the disease epidemic observed in Tasmania, comparisons of pathogenicity with isolates from south-eastern and Western Australia suggest that the Tasmanian isolates were not significantly more pathogenic (Yuan and Mohammed, 2000). Therefore, rough bark does appear to provide a micro-environment which is conducive to pathogen survival, thus increasing the likelihood of pathogen invasion under ideal environmental conditions. In the current study, bark characteristics did not differ between provenances at two-years of age and were not studied during the current field trial. Further investigation into the role of bark characteristics is required in order to ascertain whether it is an important factor in determining the susceptibility of *E. globulus* species to *En. eucalypti*.

Tree age and stem diameter have also been shown to influence the susceptibility of the host, with smaller stems (and younger trees) more susceptible to infection (Old *et al.*, 1986). Old *et al*. (1986) observed that inoculation of pole-sized *Eucalyptus delegatensis* and *Corymbia maculata* with *En. eucalypti* isolates collected from eucalypt cankers in south-eastern Australia did not cause perennial cankers or girdling of the stems. However, the same isolates of *En. eucalypti* were pathogenic to 10-month-old seedlings of *E. delegatensis, E. regnans, E. grandis, E. saligna* and *Co. maculata* (Old *et al*., 1986). In the current study, there was no association between stem diameter and disease development. Provenance B had a larger stem diameter than the other provenances, however when inoculated with *En. eucalypti*, it developed the second largest lesions. It may have been expected that provenance B would be more

resistant to girdling by the pathogen, as previous studies have indicated that thicker stems are less likely to be girdled by *En. eucalypti* (Old *et al.*, 1986). However, it is likely that in the study by Old *et al.* (1986) tree age, rather than stem diameter, was the major factor which determined host susceptibility to *En. eucalypti*. In addition, other factors may contribute to the susceptibility of trees with stem diameters of varied size. Thicker stems may be the result of a faster growing tree or thicker bark. Faster growing trees may also partition their resources into growth rather than host defence, whereas slower growing trees may expend more resources into protecting against pathogen challenge. The impact of tree growth rate on the susceptibility to *En. eucalypti* has not yet been investigated.

The ability of the host to close wounds through callus production has been linked with the relative susceptibility of *E. grandis* clones to infection by *C. cubensis* (van Zyl and Wingfield, 1999). The susceptibility of twenty-five *E. grandis* clones to *C. cubensis* was investigated in the field and the results indicated that highly tolerant clones closed wounds significantly faster (in the presence or absence of a pathogen) than highly susceptible clones. Previous studies on peach branches have found that most rapid wound closure occurred in the cultivars most resistant to the fungal canker pathogen *Leucostoma* sp. (Wensley, 1966). Future studies investigating the rate of wound healing for the various *E. globulus* provenances are required as the rate of wound closure may be an effective and practical method of rapidly assessing the susceptibility of *E. globulus* provenances to canker fungi.

The classification of the pathogenicity of *En. eucalypti* isolates is dependent upon the host provenance/species and the environmental conditions to which the host is exposed. In the current study, a significant interaction between fungal isolate and *E. globulus* provenance was noted in the glasshouse and an interaction between *E. globulus* provenance and plantation location was observed in the field. Previous studies have also reported an interaction between host species and fungal isolate (Old *et al.*, 1986) and host species and plantation location (van Heerden and Wingfield, 2002). These interactions further complicate the selection of suitable provenances for future plantings. Therefore, screening provenances for resistance to *En. eucalypti* should be conducted in the location(s) where the future plantings are proposed. In addition, the variation in pathogenicity between the *En. eucalypti* isolates indicates that screening a significant number of isolates on a range of *E. globulus* provenances is also required for a good indication of host susceptibility.

At present, the main method to control *En. eucalypti* in *E. globulus* plantations in southwestern Australia, and elsewhere in the world, is through the selection of more resistant provenances. Observation from the current study suggest that provenance characteristics, such as bark roughness and rate of wound closure, influence the susceptibility of *E. globulus* to *En. eucalypti.* This study also suggests a link between plantation location and host provenance in determining susceptibility to *En. eucalypti* in the WA plantation environment. As the two plantation locations had different soil types, rainfall and evaporation levels, it is suggested that the variation in the susceptibility of the provenances between plantation locations was due to environmental conditions of the site. Further screening of *E. globulus* provenances across a greater number of plantations, thus encompassing a range of environmental conditions (soil types, rainfall, evaporation, insect and disease levels), is required to determine whether there is a link between certain environmental conditions, such as soil moisture, and susceptibility to *En. eucalypti.* The environmental conditions which limit the stress to each provenance will then need to be considered when establishing *E. globulus* provenances at a particular location.

As *En. eucalypti* is considered a wound pathogen (Fraser and Davison, 1985) and the rate of wound closure has been identified as a key factor in host susceptibility to canker fungi, Chapter 5 investigates the impact of wounding in assessing the pathogenicity of *E. globulus* to *En. eucalypti*.

Chapter 5

A comparison of wounding and non-wounding methods of inoculation to determine the pathogenicity of *Endothiella eucalypti* to *Eucalyptus globulus*

INTRODUCTION

An understanding of the conditions under which a fungal pathogen has the potential to cause disease is vital in developing control strategies, especially within a plantation environment. Canker fungi are predominantly classed as wound pathogens, which are unable to infect a plant unless the plant has been damaged. These fungi are often associated with bark openings resulting from mechanical damage, branch stubs, leaf scars, insect damage or bark splits which provide entry points for invasion (Old and Davison, 2000; Fraser and Davison, 1985). In addition, wounds resulting from 'green pruning' (the removal of live branches at a young age) have been associated with invasion by opportunistic canker-causing fungi in *Eucalyptus nitens* plantations (Barry *et al.*, 2002). The outcome of the host-fungus interaction then depends on the ability of the fungi to invade beyond the wounded tissue and the speed at which the host responds to both wounding and pathogen invasion.

The pathogenicity of a canker fungus is determined by its ability to penetrate and colonise the host tissue. For example, a fungal species which is considered to be highly pathogenic when inoculated into a host stem, may be incapable of penetrating the stem without a wound (such as an insect sting). However, the three inoculation methods most commonly used to test the pathogenicity of fungal pathogens *in planta* involve wounding the host tissue. The most common of these methods involves removing a section of the stem with a cork borer to the depth of the cambium and filling the subsequent wound with agar containing fungal mycelia (van Zyl and Wingfield, 1999; Bunny *et al.,* 1995; Van der Westhuizen *et al.*, 1993; Jacobi, 1992) or bran colonised by fungal mycelia (Old and Kobayashi, 1988). The second method of wound inoculation involves making a shallow incision through the periderm to the phloem and inserting either an inert membrane bearing mycelia (Jackson, 1997; Old *et al.*, 1986) or a disc of colonised agar (Marks and Smith, 1990). The final method involves mechanically wounding the stem with a sharp object (e.g. scraping the surface of the stem) before placing a disc of colonised agar onto the wounded stem (van Zyl and Wingfield, 1999).

Although wound inoculation is usually employed to determine the pathogenicity of canker fungi, infection by some canker pathogens has been shown to occur through stomata and lenticels (Old and Davison, 2000).

Previous studies have concluded that *Cryphonectria eucalypti* and its anamorph, *Endothiella eucalypti* are opportunistic pathogens which require wounding to allow them to penetrate the host (Old and Davison, 2000; Yuan and Mohammed, 2000; Old *et al.*, 1990). However, to date no studies have been undertaken to examine the ability of *En. eucalypti* to cause disease in a healthy host in the presence or absence of a wound. The aim of the current study was to ascertain the ability of four *En. eucalypti* isolates, of differing pathogenicity determined by wound inoculation (Chapter 3), to cause disease in the stems of healthy *E. globulus* when inoculated via wounding and non-wounding methods.

MATERIALS AND METHODS

Experimental design

Two-year-old *E. globulus* saplings were arranged in a randomised complete block design (Steel and Torrie, 1986), and subjected to three inoculation treatments, (i) wounding, (ii) dry non-wounding and (iii) wet non-wounding. One stem from each plant was inoculated with one of the four *En. eucalypti* isolates (A17, E81, F17 and G77) or a sterile Miracloth™ disc (in the case of the control treatments), with five replicate plants per treatment. Lesion extension and colonisation were measured after five, seven and eight weeks for wounded, dry non-wounded and wet non-wounded plants, respectively (see 'Harvest' section below).

Biological materials

Eucalyptus globulus saplings supplied by ITC Ltd. were grown in 150 mm free-draining plastic pots containing potting mix (Chapter 3). The experiment was conducted in an evaporatively cooled glasshouse 20 - 27 ˚C (min-max) in late summer (February) and plants were watered overhead twice daily for 20 minutes. Plants were fertilized with a six month slow-release fertiliser (Scotts Osmocote Plus, Scotts Europe BV, Heerlen. The Netherlands.) (5 g per pot) and Isobutylidene diurea (IBDU - Mitsubishi Chemical Corp, Chiyodaku, Tokyo Japan) (2 g per pot). At the time of the experiment the plants had an average stem diameter of 10 mm at the point of inoculation (200 mm from base of plant).

The four *En. eucalypti* isolates, A17 (Albany), E81 (Manjimup), F17 (Esperance) and G77 (Denmark) (Table 3.5) were maintained on 1/2PDA plates at 24 ˚C under continuous near blue light. All isolates were passaged through a healthy *E. globulus* host (Chapter 3) immediately prior to use in the current experiment. Actively growing colonies of each isolate were subcultured onto plates containing Miracloth™ discs as described in Chapter 3.

Inoculation

The main stem of each *E. globulus* plant was inoculated using one of three inoculation methods. Stems were inoculated approximately 200 mm up from the base of the stem. In each inoculation method, sterile non-colonised discs were used as controls. The inoculation methods employed are outlined below.

Method 1 - Wound Inoculation

An *En. eucalypti* colonised disc (5 mm diameter) or sterile disc was wound inoculated into stems of *E. globulus* seedlings as outlined in Chapter 3.

Method 2 - Dry non-wounding

Stems were surface sterilised with 70% ethanol (sprayed on the stem surface), allowed to dry and an *En. eucalypti* colonised disc or sterile disc placed directly onto the stem (mycelial side touching the stem) (Figure 5.1A). The disc was held in place with Parafilm® and covered with plastic flagging tape (Figure 5.1B).

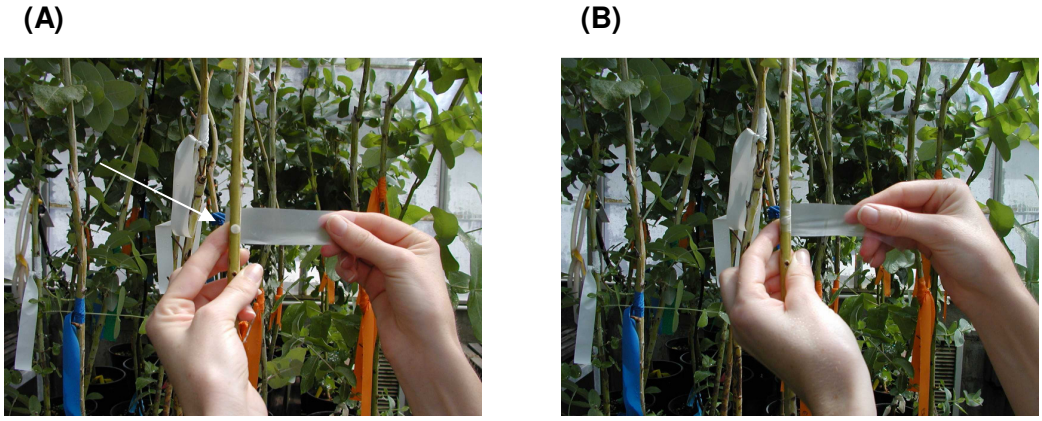


Figure 5.1 Inoculation of *Endothiella eucalypti* onto *Eucalyptus globulus* stems using the dry non-wounding method. The colonised material disk is (A) placed directly onto the stem and (B) held in place with Parafilm®.

Method 3 - Wet non-wounding

Seven days prior to inoculation, the stems were surface sterilised with 70% ethanol (as above) and a wet sterile cotton wool ball secured to the stem with Parafilm® (Lucas *et al.*, 2002). The stems were then wrapped in flagging tape to prevent desiccation. After seven days, the cotton wool was removed from the stems and an *En. eucalypti* colonised disc was placed on the stem (mycelial side touching the stem) (Figure 5.2A). The disc was then covered with a new wet sterile cotton wool ball and secured to the stem with Parafilm® and tape (Figure 5.2C, D and E) for the duration of the experiment.

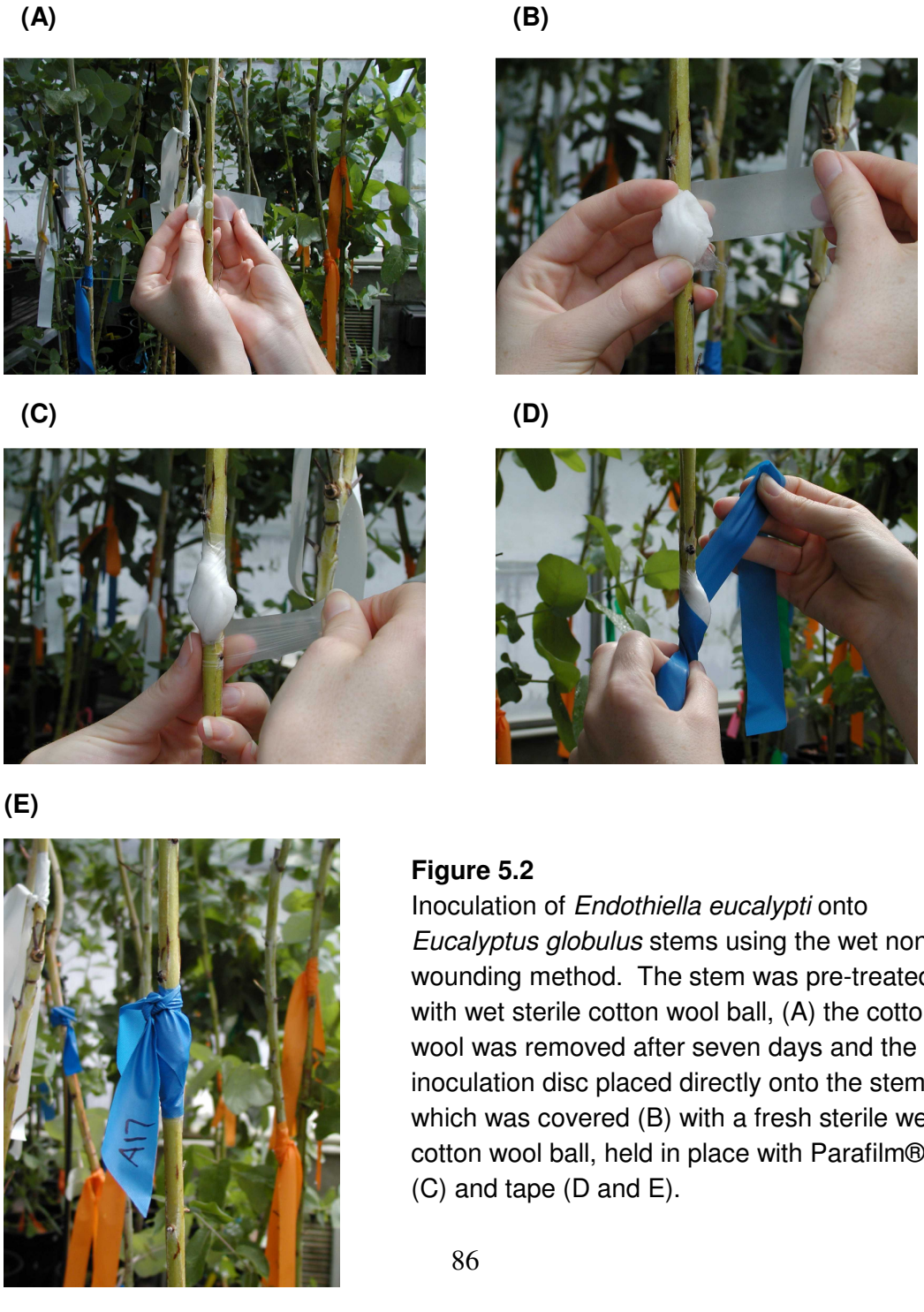


Figure 5.2
Inoculation of *Endothiella eucalypti* onto *Eucalyptus globulus* stems using the wet non-wounding method. The stem was pre-treated with wet sterile cotton wool ball, (A) the cotton wool was removed after seven days and the inoculation disc placed directly onto the stem which was covered (B) with a fresh sterile wet cotton wool ball, held in place with Parafilm® (C) and tape (D and E).

Harvest

Harvest times were staggered due to different rates of lesion development for the three inoculation methods. The wound-inoculated stems were harvested five weeks after inoculation, whilst the dry non-wounded and wet non-wounded stems were harvested seven and eight weeks after inoculation, respectively. This staggered harvesting was necessary to ensure accurate assessment of pathogen colonisation of the stem before plant death.

At harvest, lesions were measured and the stems cut into seven 10 mm segments. The first segment contained the lesion front and the remainder extending 60 mm beyond the lesion front. The 10 mm stem segment at the point of inoculation was visually examined for changes in stem appearance. Segments (10 mm) were plated onto 1/2PDA+S plates as outlined in Chapter 3. Fungal growth was monitored from each segment over seven days and total colonisation beyond the lesion determined. To check the viability of the inoculum source, the inoculum disc was recovered from each of the stems and plated onto 1/2PDA+S.

Statistical analysis

Lesion extension (mm/week) and colonisation beyond the lesion (mm/week) were analysed by ANOVA using Statistica Version 4.1 (StatSoft® Inc., OK, USA). Data were assessed for homogeneity, variation of the mean from the variance, fit to a normal distribution and log transformed to meet statistical requirements. Means were compared by LSD ($p \leq 0.01$) and are presented with standard error of the mean.

RESULTS

Observations

Each of the four *En. eucalypti* isolates produced lesions in *E. globulus* stems when inoculated using the wounding method. Pycnidia were present in at least one replicate stem for each *En. eucalypti* isolate inoculated using the wounding method and were often associated with wilting and girdling of the stem. Isolate E81 girdled all stems, followed by isolates F17, G77 and A17 girdling 80, 60 and 40 % of the stems, respectively. Plants inoculated with isolates E81 and A17 wilted in 40 % and 20 % of plants, respectively. Coppicing was observed in one plant girdled by isolate E81, gummosis was associated with another girdled stem and one plant was killed as a result of inoculation with this isolate.

Isolates E81, A17 and F17 formed lesions in all the *E. globulus* stems inoculated using the dry non-wounding method. Sixty percent of stems inoculated with isolate G77 formed lesions. The extent of stem girdling by the fungal isolates was lower in this method than observed in the wounding inoculation method. Isolate E81 killed 40% of the plants subjected to the dry non-wounding inoculation and another 20% of the seedlings wilted as a result of stem girdling at the time of harvest. Dry non-wounding inoculation with isolate F17 resulted in the death of 20% of the plants.

Isolate A17 was the only isolate to induce lesions when inoculated onto *E. globulus* stems using the wet non-wounding inoculation method. During the course of the trial, the majority of the cotton wool surrounding the inoculation disc remained moist, however, where the cotton wool had dried, lesion development increased. For example, isolate E81 induced lesions which extended 15.8 mm/ week where the cotton wool had dried (outlier data omitted) compared to no lesion where the cotton wool remained moist. This method of inoculation did not result in the girdling of any of the *E. globulus* stems. Black gummosis spots were observed under the cotton wool for stems inoculated with isolate E81 (60%), G77 (40%) as well as F17 (20%) and the control plants (20%). No other change in stem morphology was observed in control plants.

Lesion development

The correlation between lesion extension (mm/week) and total colonisation (mm/week) was highly significant (r=0.999) (Figure 5.3). Therefore, the results are discussed as lesion extension. There was a highly significant (p=0.001) interaction between inoculation method and fungal isolate (Table 5.1; Figure 5.4). Therefore, the pathogenicity of an *En. eucalypti* isolate to *E. globulus* was influenced by the method of inoculation. The inoculation method had a significant ($p<0.0001$) effect on lesion extension (Table 5.1). Overall, the wounding inoculation method produced the largest lesions followed by the dry non-wounding method, however this difference was not significant (p=0.124). Lesions formed with these methods were significantly ($p<0.0001$) larger than lesions resulting from the wet non-wounding inoculation method. Isolate A17 was the only isolate to cause lesions when inoculated using the wet non-wounding method (Figure 5.5). The *En. eucalypti* isolate had a significant (p=0.01) effect on the extent of lesion development for all three inoculation methods (Table 5.1).

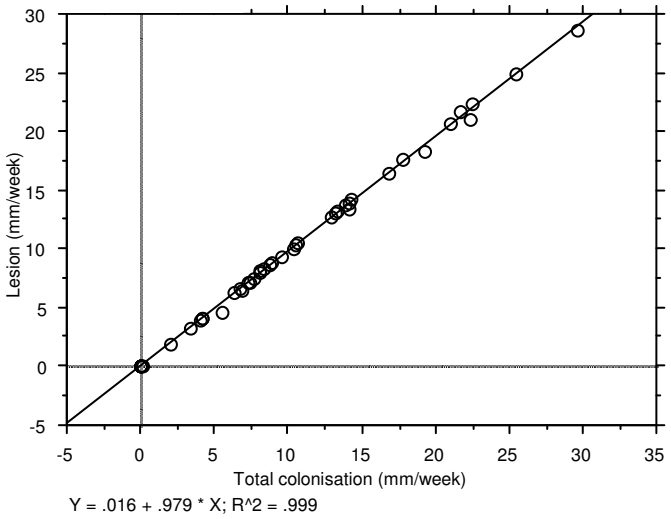


Figure 5.3
Correlation between lesion extension (mm/week) and total colonisation (mm/week) in stems of *Eucalyptus globulus* inoculated with four *Endothiella eucalypti* isolates (F17, A17, G77 and E81) using either a wounding or non-wounding method of inoculation.

Table 5.1 ANOVA of Log lesion extension (mm/week) in stems of *Eucalyptus globulus* after inoculation with *Endothiella eucalypti*. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Inoculation method (1)	20.608	0.235	87.536 (2, 38)	**<0.0001**
Isolate (2)	0.986	0.235	4.188 (3, 38)	**0.010**
1 x 2	1.111	0.235	4.721 (6, 38)	**0.001**

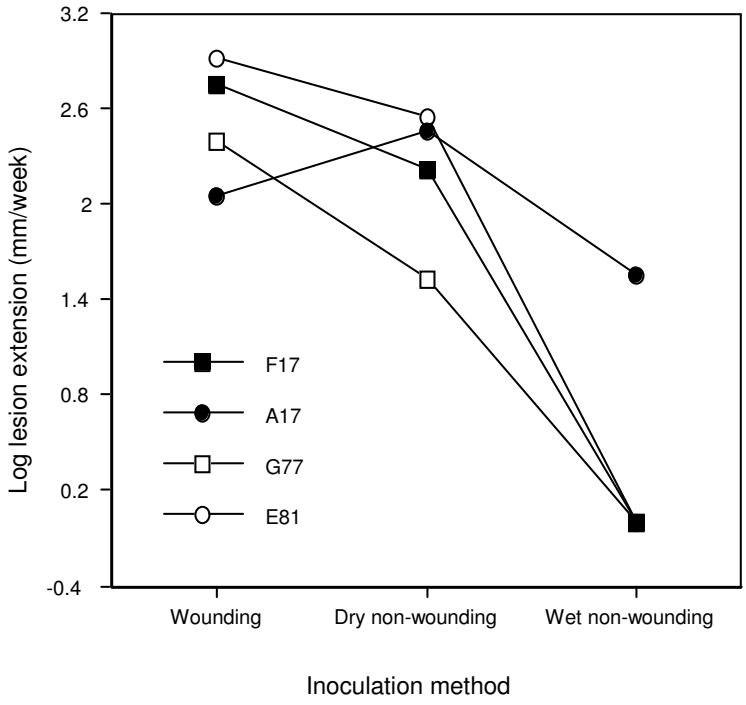


Figure 5.4 Two-way interaction plot (f (6, 38)=4.72: p<0.001) for Log lesion extension (mm/week) in stems of *Eucalyptus globulus* inoculated with four *Endothiella eucalypti* isolates (F17, A17, G77 and E81) using wounding or wet and dry non-wounding methods of inoculation.

Isolate E81 was the most pathogenic, followed by isolates F17, G77 and A17 when inoculated into stems of *E. globulus* using the wounding inoculation method (Figure 5.5). After isolate E81, A17 caused the second largest lesions when inoculated into stems using the dry non-wounding method followed by F17 and G77. Isolate A17 was the only isolate to cause lesions using the wet non-wounding inoculation method (Figure 5.5) and these extended up to 2 cm beyond the lesion front.

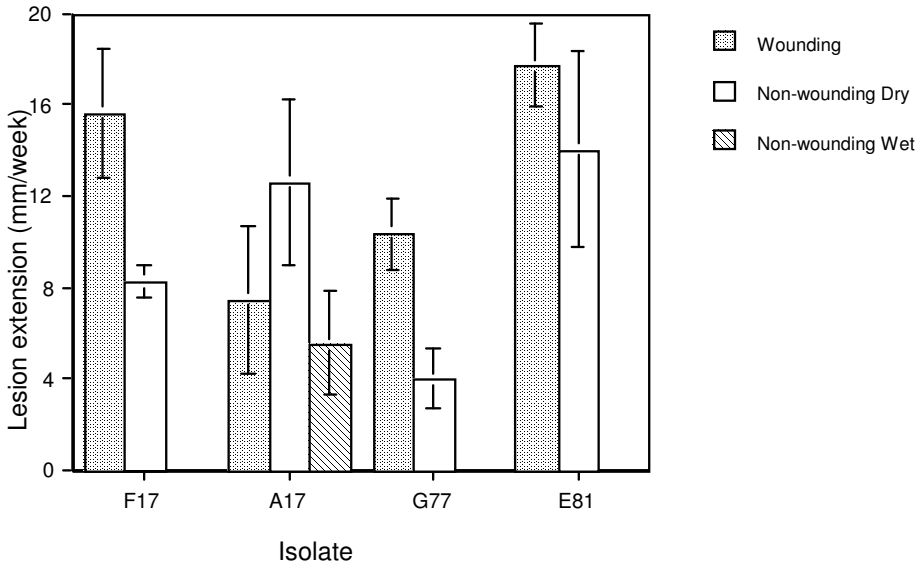


Figure 5.5 Lesion extension (mm/week) in stems of *Eucalyptus globulus* inoculated with *Endothiella eucalypti* isolates F17, A17, G77 and E81, using wounding or wet and dry non-wounding methods of inoculation. Bars indicate standard errors of the mean.

There was no correlation between stem diameter and lesion extension or total colonisation (mm/ week) in any of the inoculation methods (r=0.06 and r=0.05, respectively) (data not shown).

In the current study, the pathogenicity of the four *En. eucalypti* isolates varied depending on the method of inoculation used. For example, isolate A17 was the least pathogenic isolate when inoculated into the stem via a wound. However, it was the second most pathogenic isolate when inoculated onto the *E. globulus* stems using the dry non-wounding inoculation method (Figure 5.5). Therefore, pathogenicity of isolates was rated according to the inoculation method employed (Table 5.2).

Table 5.2 Pathogenicity of four *Endothiella eucalypti* based on the wounding and the dry non-wounding methods of inoculation in stems of *Eucalyptus globulus*.

Isolate	Collection Location	Pathogenicity rating* (wounding)	Pathogenicity rating* (dry non-wounding)
A17	Albany	weakly pathogenic	moderately pathogenic
G77	Denmark	mildly pathogenic	weakly pathogenic
F17	Esperance	moderately pathogenic	mildly pathogenic
E81	Manjimup	highly pathogenic	highly pathogenic

***Pathogenicity ratings**
Weakly Pathogenic - occasionally causes lesions, lesions superficial and easily walled off.
Mildly Pathogenic - causes lesions, lesions superficial, pycnidia rarely present
Moderately pathogenic - always causes lesions, lesions penetrating into xylem, some girdling, pycnidia often present
Highly pathogenic - always causes lesions, lesions extensive leading to girdling, with gummosis and pycnidia often present.

DISCUSSION

For the first time, this study has shown that *En. eucalypti,* previously considered an opportunistic pathogen requiring a wound for host invasion, is capable of causing disease in intact stems of healthy *E. globulus*. Although inoculating isolates of *En. eucalypti* into *E. globulus* stems using the wounding method resulted in the largest lesions, inoculating *En. eucalypti* onto dry intact stems also caused lesions.

These results indicate that wounding the stem during the inoculation process affects the susceptibility of the host to the *En. eucalypti* isolate. For example, isolate A17 caused the smallest lesions when inoculated into a wounded stem, however it induced the second largest lesions when inoculated onto a dry non-wounded stem and was the only isolate to cause lesions using the wet non-wounded inoculation method. When the periderm is damaged and the underlying phloem is exposed to the air, there are rapid cellular and tissue responses, such as the accumulation of lignin and suberin, that result in the formation of a new periderm (Old and Davison, 2000). Wounding alone has been found to stimulate defence enzymes in resistant plants, priming the host against infection by the pathogen (Old and Davison, 2000; Bucciarelli *et al.*, 1998). Therefore, the growth of *En. eucalypti* isolate A17 may have been inhibited by host wound responses when introduced onto a wounded surface. In comparison, the presence of a wound did not alter the relative pathogenicity of isolate E81 which caused the largest lesions in *E. globulus* stems when inoculated using the wounding and dry non-wounding methods. However, this isolate was unable to cause lesions when inoculated using the wet non-wounding method. Isolates E81, G77 and F17 were less able to penetrate non-wounded stem tissue than isolate A17. However, once within the host

they were more effective in penetrating the host's defences at the wound site and colonising host tissue. Therefore, the pathogenicity of a fungal isolate under field conditions, may be attributed to a combination of (i) the ability of the isolate to invade non-wounded tissue and (ii) the interaction between the pathogen and host at the wound surface. Further studies into the host defence responses to inoculation with and without wounding are required in order to determine whether wound inoculation 'primes' the *E. globulus* host against future pathogen challenge, thereby increasing the resistance of the host and biasing pathogenicity trials.

The rate of wound closure has been identified as a factor which impacts on the severity of disease caused by canker fungi. As mentioned previously (Chapter 4), a wounding study conducted by van Zyl and Wingfield (1999) concluded that the tolerance of *E. grandis* clones to the canker pathogen *C. cubensis* was directly proportional to the rate at which the host healed wounds. In comparison, a study on resistant and susceptible *Populus tremuloides* indicated that the morphological response (in the formation of callus) of both genotypes to wounding was identical (Bucciarelli *et al.*, 1998). The size of the wound has also been identified as a factor affecting the severity of cankers in *E. grandis* clones (van Zyl and Wingfield, 1999). This study reported that small wounds provided greater opportunity for lesion development, whilst larger wounds result in heightened levels of host response thus limiting pathogen invasion. Although the response to wounding can vary between and within species, as well as according to the size of the wound, the results of the current and previous studies (Barry *et al.*, 2002; van Zyl and Wingfield, 1999; Bucciarelli *et al.*, 1998) indicate that the type of wound inoculation may influence the extent of disease development.

When the periderm is damaged and the underlying tissues are invaded by a pathogen, the response of the host may be similar to the response to wounding (Old and Davison, 2000). However, wounding alone does not stimulate the host defence responses to the same extent as the presence of the pathogen. Studies by Barry *et al.* (2002) recorded less phenolic production and discolouration in wounds of two-year-old *E. nitens* inoculated with sterile agar compared to fungal inoculum. Furthermore, the variation in the degree of host response was attributed to the aggressiveness of the fungi, with more aggressive fungi continually challenging the host and consequently defence mechanisms constantly being elicited (Barry *et al.*, 2002). Although the response of the host to wounding is not as intense as its response to pathogen challenge, the combination of a concentration of compounds such as phytoalexins, lignin and suberin

at the wound site and the stimulation of the host defence mechanisms in response to the wound, may play an important role in reducing damage caused by canker fungi.

The season in which wounds are created have been shown to influence the susceptibility of the host to infection (Barry *et al.*, 2002; Mohammed *et al.*, 2000). For example, differences in rainfall and temperature may influence the hydraulic status of the xylem, rendering the host more or less compromised by wounding, particularly in the case of wood rotting fungi (Barry *et al.,* 2002). Environmental factors may influence the distribution of resources, affect phenol production and result in changes in disease susceptibility. Studies conducted on *Corymbia maculata* in mainland Australia showed that the decrease in fungal decay in summer was correlated with increased phenol production (Mireku and Wilkes, 1989). In contrast, studies on plantation-grown *E. nitens* in Tasmania found no evidence that decay was more serious following wounding at any time of the year. However, the number of decay lesions in the sapwood associated with wounds pruned in spring and summer were slightly higher compared with those pruned in autumn and winter (Mohammed *et al.*, 2000). The activity of the pathogen in winter compared to summer has also been shown to play a role in wound infection. For example, it has been shown that *Diplodia pinea* grown in pine plantations in South Africa produce less spores in winter than in summer (Roux *pers. comm.*). Therefore the response to wounding at various times of the year will differ according to the climatic conditions of the region, the type of host and pathogen and the suitability of the host to the environmental conditions.

It has been suggested that pre-treatment of the *E. globulus* stems with moist cotton wool prior to introduction of the fungus creates an ideal environment for the invading pathogen (Lucas *et al.*, 2002). In the current study, only one *En. eucalypti* isolate (A17) caused lesions when inoculated into *E. globulus* stems using the wet non-wounding method. However, in a small number of replicates where the cotton wool balls fastened to the stems were inadvertently allowed to dry after moist pre-treatment, lesion development (for some isolates) was equivalent to that of the wound inoculation method. Therefore, moist pre-treatment of *E. globulus* stems may facilitate the penetration of *En. eucalypti* if the stem is dry at the time of pathogen introduction. These finding are in contrast with those of Lucas *et al.* (2002), who showed that *Phytophthora cinnamomi* colonised the stems of *E. marginata* seedlings with periderm more effectively if moist pre-treatment was followed by introduction of the fungus under moist conditions. As the studies on *E. marginata* examined the response of

P. cinnamomi, a water mould, the continuously high moisture environment may have been unsuitable for *En. eucalypti,* which resulted in reduced disease development. Further studies investigating the processes involved in the infection of *E. globulus* with *En. eucalypti* are required to establish the conditions under which infection is most likely to occur.

The green bark (periderm) of the *E. globulus* stems was thin (approximately 2 mm in diameter) and smooth in appearance, with no visible cracking. Therefore, it may have been easily penetrated by a more aggressive pathogen, even in the absence of entrance points, such as cracks or insect wounds. This was illustrated in the current study with E81, the most pathogenic *En. eucalypti* isolate, which caused the largest lesions whether inoculated in the presence or the absence of a wound. However, as the trees age the green periderm of young stems is strengthened with the deposition of phellem layers, which could provide greater protection from invasion by *En. eucalypti*. Therefore, the results of the current study may not apply to older trees. Further research examining the role of bark thickness in association with tree age and the subsequent susceptibility to penetration by *En. eucalypti,* is required to determine whether the ability of *En. eucalypti* to penetrate non-wounded tissue is restricted to young trees with thin green bark.

The patterns in isolate pathogenicity for the current study and that of Chapters 3 and 4 were similar, with the exception of isolate F17. Although lesion extension recorded for the *En. eucalypti* isolates differed slightly between experiments (Chapters 3, 4 and 5), isolate E81 was consistently the most pathogenic isolate when wound inoculated. *Eucalyptus globulus* stems inoculated with isolate F17 developed average lesion extension of 16.11 mm and 15.5 mm per week in studies reported in Chapters 4 and 5, respectively. However, it did not induce lesions in the experiment conducted as part of Chapter 3. Although the experiments were conducted in the same temperature-controlled glasshouse, factors such as slight changes in environmental conditions, differences in tree age (one-year-old trees used in Chapter 3 compared to two-year-old trees used in Chapters 4 and 5) and host provenance (Chapter 3 used trees of a different provenance than Chapters 4 and 5) may have contributed to differences in isolate pathogenicity.

In conclusion, the current study illustrated that *En. eucalypti* was capable of causing disease in healthy *E. globulus,* even in the absence of a wound. A variation in the

development of disease following inoculation with some *En. eucalypti* isolates was noted between wounding and non-wounding inoculation techniques. Therefore, a combination of wounding and non-wounding methods of inoculation may be required in order to assess the ability of an *En. eucalypti* isolate to cause disease in healthy *E. globulus*. Further knowledge of the infection process of *En. eucalypti,* the response of *E. globulus* to mechanical wounding and the environmental conditions which facilitate the development of disease will aid in developing management strategies for the control of *En. eucalypti* in the plantation environment, particularly in relation to 'green pruning'.

Chapter 6

Genotypic diversity of *Endothiella eucalypti* isolated from *Eucalyptus globulus* plantations in southwestern Australia

INTRODUCTION

Variability in colony morphology and pathogenicity within the WA population of *Endothiella eucalypti* (Chapters 2, 3 and 4) suggests a diverse population, although to date only the asexual stage of *Cryphonectria eucalypti* has been observed in the state (Paap, 2001; Davison and Coates, 1991). The genotypic diversity within *En. eucalypti* populations associated with *Eucalyptus globulus* in WA has not been studied. Therefore, the aim of the current study was to investigate the genotypic diversity within a WA population of *En. eucalypti* using methods of vegetative compatibility (VC) grouping, a traditional method of assessment, and DNA fingerprinting.

Vegetative incompatibility is a phenotypic characteristic that has been widely used to study genotypic diversity in fungi (Burgess *et al*., 2001; van Heerden and Wingfield, 2001; Cortesi and Milgroom, 1998; Couteaudier and Viaud, 1997). Genotypic diversity is an informative measure of the genetic structure of a population (Stoddart and Taylor, 1988). If isolates are incompatible, a mycelial boundary or exclusion zone will be present between the two competing isolates. If isolates are compatible, the mycelia of the two isolates will grow together and the boundary between the isolates becomes indistinguishable. A study of the genotypic diversity of *Endothiella* associated with *Corymbia calophylla* in southwestern Australia using VC analysis indicated a high degree of genotypic diversity (Paap, 2001).

Vegetative compatibility assessment is an indirect indication of genotypic diversity which is implied from a phenotypic characteristic (i.e. the formation of barrage zone). Therefore, in the past decade, molecular methods have been used to more accurately assess genotypic variation. Recently, Australian isolates of *En. eucalypti* (anamorph of *C. eucalypti*) have been closely linked to the South African *C. eucalypti* using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis (Venter *et al.*, 2001). This method was also used, in association with morphological characteristics, to propose a new morphotype among Canadian *Sphaeropsis sapinea* (now *Diplodia pinea*, De Wet *et al.*, 2003) isolates (Hausner *et al*.,

1999). The use of molecular techniques, such as RFLP's, allows more detailed assessment of diversity within a population at the genetic level.

Inter-simple sequence repeat (ISSR) analysis, previously termed randomly amplified microsatellite (RAM) analysis (Gherbi *et al*., 1999), was chosen for use in the current study as it is a relatively inexpensive, fast and repeatable method of assessing the diversity within the WA *En. eucalypti* population. The ISSR-PCR method involves PCR amplification of regions between adjacent, inversely oriented microsatellites (tandem arrays of short repeats of a few nucleotides) using a simple sequence repeat (SSR)-containing primer (Blair *et al.*, 1999). Microsatellite-anchored primers that anneal to an SSR region can amplify regions between adjacent SSRs (inter-simple sequence repeats; ISSR). DNA fingerprinting with ISSR-PCR provides superior discrimination among individual fungal isolates due to analysis of a large number of fragments throughout the genome (Grunig *et al.*, 2001) and the ISSR markers are useful in detecting genetic polymorphisms (Nagaoka and Ogihara, 1997).

The specific aims of the current study were (i) to test the suitability and reproducibility of ISSR-PCR in detecting variation within the WA population of *En. eucalypti,* (ii) to make comparisons with isolates of *C. eucalypti* (collected from South Africa and Queensland) and *C. cubensis* (from China) with the WA *En. eucalypti* population; and (iii) to compare the use of vegetative compatibility, a more conventional form of assessing population diversity, with more recent molecular techniques.

MATERIALS AND METHODS

Vegetative compatibility

Experimental design

To determine the genetic diversity within and between *En. eucalypti, C. eucalypti* and *C. cubensis* isolates, VC groups were identified on oatmeal agar with two replicate plates for each of the 30 isolates examined. As the experimental design, which ensured all possible isolate combinations, required a total of 31 isolates, isolate E81 was used twice.

Fungal isolates

Single spore cultures were prepared for 26 *En. eucalypti* isolates collected from plantations in southwestern Australia (five each from the Albany, Manjimup, Denmark and Margaret River regions, four from the Bunbury region and two from the Esperance

region), one *Endothiella* isolate collected from *Co. calophylla* in southwestern Australia, two *C. cubensis* isolates collected from *E. marginata* in WA (Department of Conservation and Land Management (DCLM) culture collection; CRY A = DCE383 and CRY B = DCE384), and one *C. eucalypti* isolate from South Africa according to the method outlined in Chapter 2 (Table 6.1). Isolates of the *Cryphonectria* spp. were used as a comparison to the *En. eucalypti* isolates identified in the current study. Single spore cultures were maintained on 1/2PDA at 20 °C under continuous near blue light for seven days. Isolates were used from other hosts and countries as a comparison with those from WA. Numbers of these comparison isolates were limited due to availability.

Preparation for determining vegetative compatibility

The medium used in this study was oatmeal agar (van Heerdan and Wingfield, 2001). Briefly, 100 g oatmeal was added to 1 L of distilled water and the mixture boiled for two hours, stirring occasionally. The oatmeal was then sieved through cheesecloth and added to pre-dissolved agar (30 g/ L) before autoclaving. Vegetative compatibility groupings were determined by placing 2 x 2 mm agar plugs from the edges of actively growing single spore cultures (7-10 days old) onto oatmeal agar (in sterile 9 cm diameter Petri dishes) in an arrangement so as each isolate interacted with the other five isolates on that plate. Six isolates were compared on a single Petri dish and across 31 plates isolates were compared in every possible combination. Plates were stored at 20 °C in the dark for seven days then under near blue light for another seven days. Plates were assessed after seven and 14 days.

Assessment of diversity

Isolates were classified into VC groups according to the method described by Anagnostakis (1977). That is, if the mycelium of two isolates had merged, they were considered vegetatively compatible. Isolates were considered incompatible if they had grown to a meeting point, but remained separated by a barrage reaction formed along the line of contact. Each VC group was assigned a number and isolates belonging to the same VC group were treated as a phenotype. The different phenotypes were then used to estimate genotypic diversity using two statistical parameters. An estimate of V/N where V = number of VC groups and N = population size was calculated. The genotypic diversity (G) was then estimated using the equation $G = 1/\Sigma pi^2$, where *pi* is the observed frequency of the genotype according to Stoddart and Taylor (1988). The maximum percentage of genotypic diversity was also determined by dividing the genotypic diversity by the total sample size (G/N).

Table 6.1 Origin and host species of fungal isolates selected for use in vegetative compatibility (VC) and inter-simple sequence repeat -PCR (ISSR-PCR) analyses.

Isolate Code	Collection location	Identification	Collected by	Host (Plantation Number Figure 2.1)	VCG	ISSR-PCR
A2	Albany	*E. eucalypti*	T. Jackson	*E. globulus* (22)	√	√
A17				*E. globulus* (22)	√	√
A83				*E. globulus* (21)	√	√
D42				*E. globulus* (20)	√	√
D32				*E. globulus* (19)	√	√
B60	Manjimup	*E. eucalypti*	T. Jackson	*E. globulus* (9)	√	√
E10				*E. globulus* (7)	√	√
E51				*E. globulus* (7)	√	√
E69				*E. globulus* (11)	√	√
E81				*E. globulus* (11)	√	√
E98				*E. globulus* (8)	x	√
F9	Esperance	*E. eucalypti*	T. Jackson	*E. globulus* (25)	√	√
F17				*E. globulus* (25)	√	√
G2	Denmark	*E. eucalypti*	T. Jackson	*E. globulus* (14)	√	√
G38				*E. globulus* (13)	√	√
G62				*E. globulus* (17)	√	√
G75				*E. globulus* (16)	√	√
G77				*E. globulus* (15)	√	√
H12	Margaret River	*E. eucalypti*	T. Jackson	*E. globulus* (6)	√	√
H15				*E. globulus* (6)	√	√
H18				*E. globulus* (6)	√	√
H25				*E. globulus* (5)	√	√
H29				*E. globulus* (5)	√	√
H51	Bunbury	*E. eucalypti*	T. Jackson	*E. globulus* (2)	√	√
H57				*E. globulus* (2)	√	√
H74				*E. globulus* (1)	√	√
H77				*E. globulus* (1)	x	√
H118				*E. globulus* (3)	√	√
CRY A	Western Australia	*C. cubensis*	E. Davison	*Eucalyptus marginata*	√	√
CRY B					√	√
PP1.19	Manjimup	*Endothiella sp.*	T.Paap	*Corymbia calophylla*	√	√
Cry 00062	Frankfort, South Africa	*C. eucalypti*	I. van der Westhuizen	NA	√	√
QLD 1	Queensland	*C. eucalypti*	G. Hardy	*Eucalyptus grandis*	x	√
QLD 22	Queensland	*C. eucalypti*	G. Hardy/ T. Burgess	*E. grandis*	x	√
QLD 23	Queensland *C. eucalypti*	*C. eucalypti*	G. Hardy/ T. Burgess	*E. grandis*	x	√
QLD 24	Queensland	*C. eucalypti*	G. Hardy/ T. Burgess	*E. grandis*	x	√
CHINA 6	China	unknown *Cryphonectria sp.*	G. Hardy/ T. Burgess	*E. grandis/ E. urophylla*	x	√
CHINA 9	China	*C. cubensis*	G. Hardy/ T. Burgess	*E. grandis/ E. urophylla*	x	√
CHINA 12	China	unknown *Cryphonectria sp.*	G. Hardy/ T. Burgess	*E. grandis/ E. urophylla*	x	√

Key

√ = present in analysis

x = not present in analysis

Inter-simple sequence repeat -Polymerase chain reaction (ISSR-PCR) analysis

Experimental Overview

Single spore cultures were prepared for 28 *En. eucalypti* isolates (Table 6.1) collected from southwestern Australia, one *C. cubensis* isolate from the DCLM (DCE 384), one *C. eucalypti* isolate from South Africa and one unknown *Endothiella* from *Co. calophylla* according to the method outlined in Chapter 2 (Table 6.1). For comparison, three *Cryphonectria* spp. isolates from China (one *C. cubensis* and two unknown) and four *C. eucalypti* from Queensland were also examined (Table 6.1). Numbers of these comparison isolates were limited due to availability. The DNA was extracted from the mycelium of each seven-day-old single spore isolates according to the method outlined in Chapter 2. The 38 fungal isolates were subject to ISSR-PCR analysis using four primers/ primer combinations. Phylogenetic trees were constructed with the software package Paup* 4.0b4a (PPC) (Phylogenetic Analysis Using Parsimony: Swofford, 1998).

Preparation of fungal isolates

Single spore isolations were conducted according to the method outlined in Chapter 2 and cultures were maintained on 1/2PDA plates at 24 ˚C under continuous near blue light for seven days. Four 5 mm^2 pieces of agar were taken from the edge of actively growing colonies (for each isolate) and placed into a microfuge tube containing malt extract broth (20 g/L malt extract; Becton-Dickson & Company, USA) and incubated at 24 ˚C in the dark with regular gentle mixing. After seven to 14 days, the microfuge tubes containing broth and fungal mycelium were centrifuged at 14 000 *g* for 10 minutes and the broth discarded. The remaining agar and mycelium was then used for DNA extraction as outlined in Chapter 2. The DNA concentrations were determined using a Hoefer DyNA Quant 200 fluorometer according to the manufacturers instructions, in conjunction with visualisation of band size and intensity of DNA electrophoresed on a 1% agarose gel as outlined in Chapter 2. The DNA was stored at –20 °C until required for ISSR-PCR analysis.

Screening of ISSR primers

Eight 5'-anchored ISSR-primers: 5'DDB $(CCA)_5$, 5'DHB $(CGA)_5$, 5'HVH$(GTG)_5$, 5'NDB$(CA)_7$C, 5'NDV$(CT)_8$, 5' HBDB$(GACA)_4$, 5'YHY$(GTG)_7$ and 5'HVD$(CAT)_5$ (van der Nest *et al*., 2000) with 64 possible primer combinations; were tested on an *En. eucalypti* isolate (F17). ISSR-PCR of the fungal isolate (F17) was conducted as

previously described (van der Nest *et al*., 2000) with the eight primers to determine the optimal annealing temperature (ranging from 37 °C to 52 °C), as well as DNA and primer concentrations. All possible primer combinations were then screened using the temperature, DNA and primer concentrations previously determined. From the 64 primer combinations examined, the following four primer or primer pairs were selected for use in the current study: 5'HVD$(CAT)_5$, 5'HVD$(CAT)_5$ and 5'NDV$(CT)_8$, 5'DHB$(CGA)_5$ and 5'HBDB$(GACA)_4$ and 5'HBDB$(GACA)_4$. ISSR-PCR was then conducted for all isolates with four primer or primer pairs, as previously described (van der Nest *et al*., 2000) with the exception of the annealing temperature that was set at 46 °C. The PCR reaction consisted of 0.15 µL of *Taq* polymerase (5.5 units µL, Biotech International), 5 µL of 5x polymerisation buffer (equivalent to 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ ml gelatin, 0.2 mM dNTPs; Biotech International), 1.5 µL of each primer and 1 µL fungal DNA (2.5 ng). The reaction mix was made up to 25 µL. The magnesium concentrations varied according to the primer/ primer pairs with CAT5 and GACA4 requiring 2 µL, CAT5 - CT8 requiring 3 µL and CGA5 - GACA4 requiring 1 µL $MgCl_2$ (25 mM; Biotech International). The reaction was carried out using a GeneAmp® PCR System 2700 (Applied Biosystems, Singapore) programmed for an initial denaturization of two minutes at 94 °C followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at the annealing temperature of 46 °C, one minute at 72 °C and a final extension of five minutes at 72 °C. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide (10 mg mL^{-1}), de-stained in distilled water and visualised under UV illumination. The concentration of the fungal DNA added to the reaction for each primer or primer pair was adjusted according to the clarity of the resultant bands. Once the DNA concentration for each isolate was optimised the reaction for each of the four primer sets was repeated.

Table 6.2 Primer sequence, melting temperature and size.

Primer	Sequence*	Melting temperature (°C)	Size (mer)
CCA	5'DDB $(CCA)_5$	53	18
CGA	5'DHB $(CGA)_5$	53	18
GTG	5'HVH$(GTG)_5$	53	18
CA	5'NDB$(CA)_7$C	49	18
CT	5'NDV$(CT)_8$	50	19
GACA	5'HBDB$(GACA)_4$	52	20
CAT	5'HVD$(CAT)_5$	42	18
GT	5'YHY$(GTG)_7$	49	18

*Coding of degenerative sites: B = G, T or C; D = G, A or T; H = A, T or C; V = G, A or C; Y = C or T; N = A, C, G or T.

Analysis of Inter-simple sequence repeat -PCR (ISSR-PCR)

For each primer set the total number of bands was established across all 38 isolates and the presence (coded 1) or absence (coded 0) of these bands, or where bands were unclear (coded N) was recorded. Parsimony analysis was performed on the data set using Paup* 4.0b4a (PPC). All characters were treated as unordered and were equally weighted. The most parsimonious trees were obtained by heuristic searches with random addition in 1 000 replicates. A majority-rule consensus tree, which consists of all groups that occur in more than 50% of the trees investigated, was determined using Paup* rooted in the unknown *Cryphonectria* isolates from China (CH6 and CH12). The bootstrap consensus tree was constructed using the same conditions as the heuristic search. The consistency index (CI) and retention index (RI) were also calculated using Paup*.

Banding profiles were then established and each of the 38 isolates classified according to the banding profile for each primer set. Isolates with the same DNA fingerprint for all primers/ primer pairs tested were considered as indistinguishable haplotypes. These haplotypes were used to describe the diversity within the WA isolates studied, the estimate of V/N, genotypic diversity (G) and maximum percentage of genotypic diversity was estimated according to Stoddart and Taylor (1988) as described previously.

RESULTS

Vegetative compatibility

After seven days, barrage zones were visible between incompatible isolates and a high level of variation between the isolates was apparent (Figure 6.1A). Incompatible interactions resulted in the formation of a barrage zone, which in some cases was raised (Figure 6.1E) and in other cases resulted in a clear or exclusion zone (Figure 6.1C and D). Incompatible interactions were often associated with production of orange pycnidia after 14 days (Figure 6.1D).

Very high genotypic diversity was recorded amongst the 28 WA *E. eucalypti* isolates with only two isolates (H12 and H18) compatible (mycelium grew together without opposition) (Figure 6.1B). The remaining 26 isolates were incompatible with one another. The *C. eucalypti* isolate from South Africa (Cry 0062) showed an incompatible reaction with the *En. eucalypti* isolates. The genotypic diversity (G) for

the total number of genotypes (VC groups) was estimated to be 24.14. The maximum percentage of genotypic diversity (G/N) was 92.8% (Table 6.3).

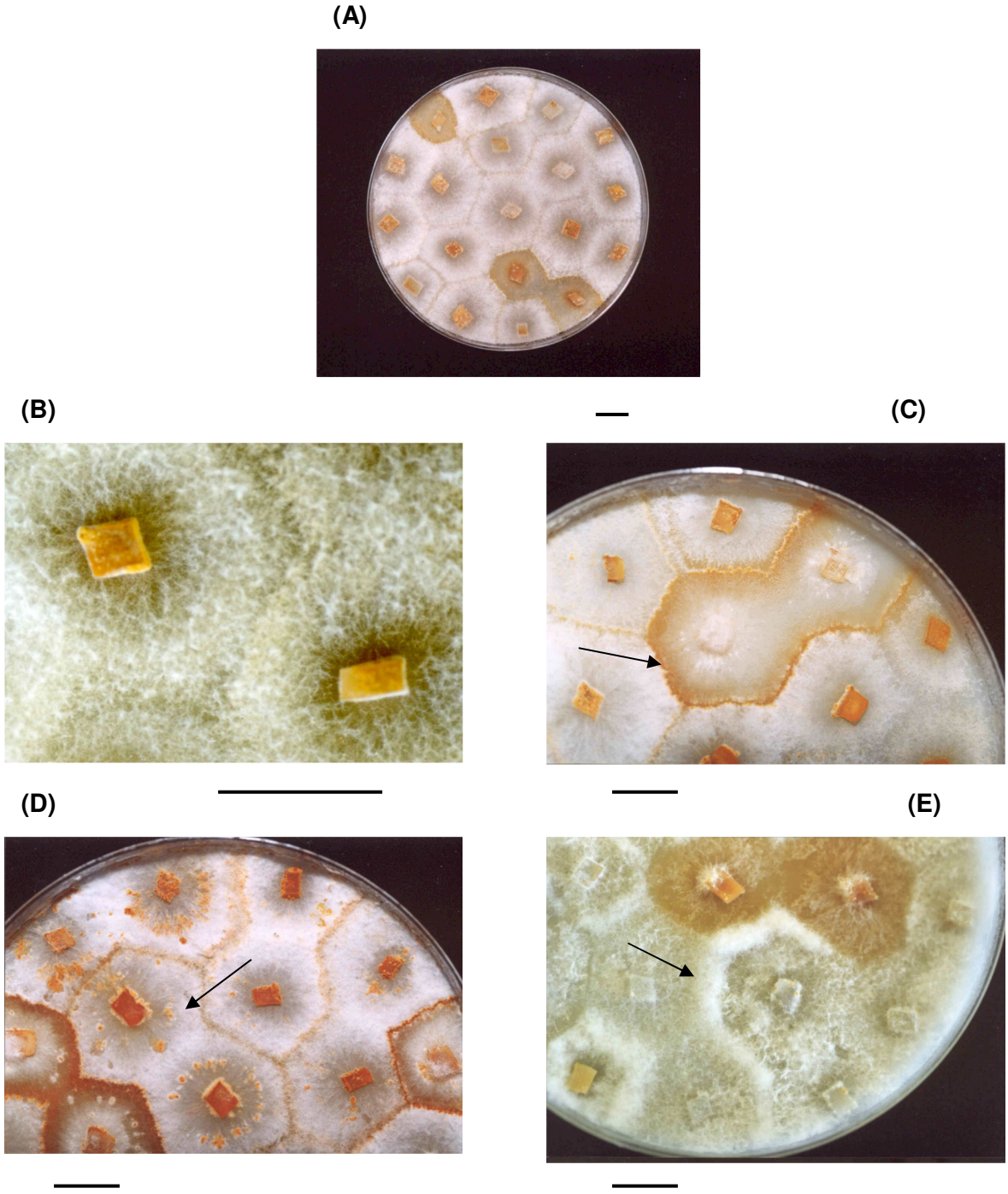


Figure 6.1 Vegetative compatibility of seven day-old isolates of *Endothiella eucalypti* grown on oatmeal agar (A), illustrating vegetative compatibility (B), and vegetative incompatibly interactions such as zones of exclusion (C and D) and raised boundary zones (E). Bar = 1 cm.

Table 6.3 Genotypic diversity in the Western Australian population of *Endothiella eucalypti* determined from vegetative compatibility.

Genotype	Frequency
24	1
1	2

Sample size: 26
Number of genotypes: 25
V/N: 0.96
Genotypic diversity (G): 24.14
Maximum percentage of genotypic diversity (G/N): 92.8%

Inter-simple sequence repeat -PCR (ISSR-PCR)

The banding pattern data for the ISSR-PCR analysis of the 38 isolates were analysed using three methods: (i) calculation of genotypic diversity using haplotype groupings; (ii) construction of banding profiles; and (iii) analysis of binary code using Paup*.

If an isolate had the same banding pattern for each of the four primer sets and it was distinctly different from any other isolates patterns, then it was considered a distinct haplotype. A total of 8 haplotypes were identified from banding patterns across the four primer sets. Genetic diversity among the WA population of *En. eucalypti* isolates indicated by inter-repeat PCR analysis was calculated at 2.54 and the maximum percentage of genotypic diversity 9.07% (Table 6.4).

Table 6.4 Genotypic diversity in the Western Australian population of *Endothiella eucalypti* calculated from inter-simple sequence repeat -PCR (ISSR-PCR) banding patterns.

Genotype	Frequency
3	1
4	2
1	17

Sample size: 28
Number of genotypes: 8
V/N: 0.28
Genotypic diversity (G): 2.54
Maximum percentage of genotypic diversity (G/N): 9.07%

For each fungal isolate, the primer sets displayed up to ten amplification products. All primer sets detected variation within the WA *En. eucalypti* population and between *C. cubensis* and *C. eucalypti* species. Banding patterns obtained by DNA fingerprinting enabled isolates to be distinguished according to number, size and intensity of fragments. From the banding patterns of the individual primer sets, a total of 32 different banding profiles was established for the 38 isolates (Figure 6.2). The isolates

were then characterised according to a banding profile (Table 6.5). The $(CAT)_5$ ISSR primer produced the clearest banding profile and was suitable for inter- and intra-species comparison. Reproducibility of the ISSR patterns was tested by amplifying DNA concentrations at dilutions of 1:5, 1:10, 1:20 and 1:40 of a selection of isolates. The final DNA concentration required for successful banding profiles for each of the primer sets varied between isolates. Although subjected to dilution analysis, the *C. cubensis* isolate from WA (Cry B) consistently failed to produce visible bands for three of the four primer sets (Table 6.5).

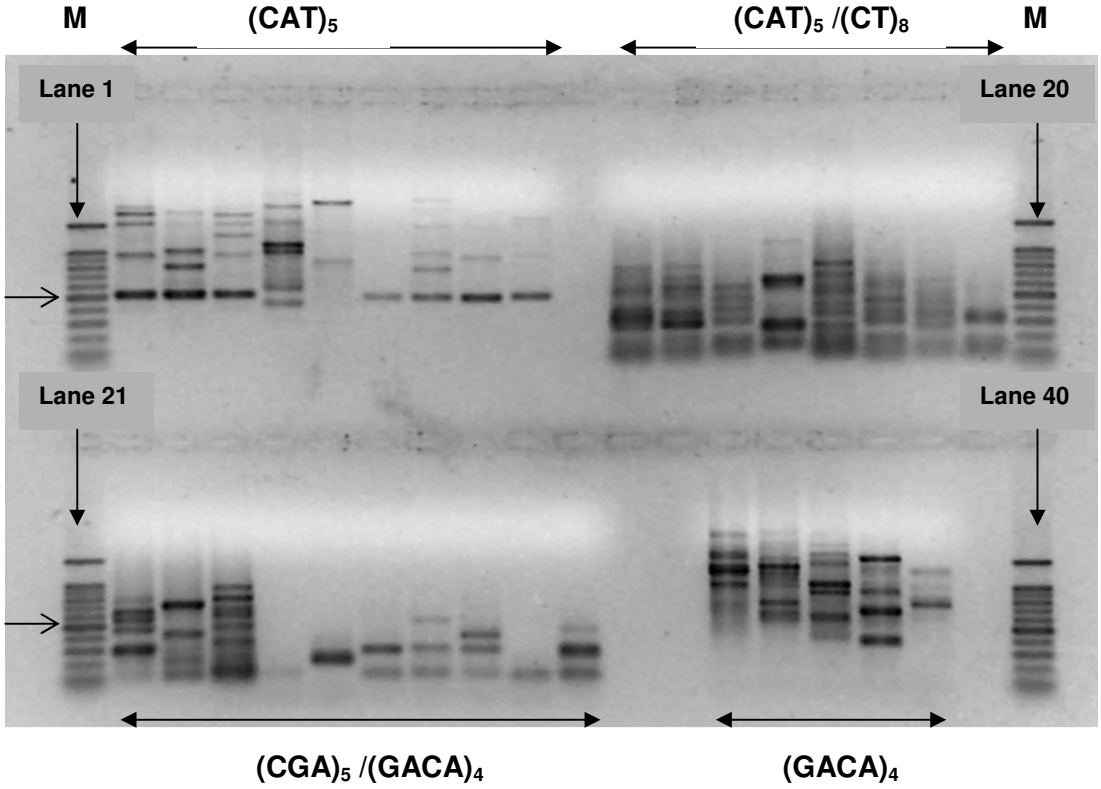


Figure 6.2 Examples of the DNA profiles established from selected isolates of *Endothiella eucalypti, Cryphonectria eucalypti* and *C. cubensis* produced by inter-simple sequence repeat PCR (ISSR-PCR). Lanes 2-10 correspond to DNA profiles 1-9 obtained using primer $(CAT)_5$, lanes 12-19 to DNA profiles 1-8 using primers $(CAT)_5$ /$(CT)_8$, lanes 22-31 to DNA profiles 1-10 using primers $(CGA)_5$ /$(GACA)_4$, and lanes 34-38 to DNA profiles 1-5 using primer $(GACA)_4$. Isolate profiles are displayed in Table 6.4. Lanes 1, 20, 21 and 40 shows the DNA molecular size markers (100 bp ladder). Arrows ($\rightarrow$) indicate 500 bp band.

Table 6.5 Classification of DNA profiles for each of the isolates of *Endothiella eucalypti*, *Cryphonectria eucalypti, C. cubensis* and unidentified *Cryphonectria* spp. according to inter-simple sequence repeat PCR (ISSR-PCR) analysis displayed in Figure 6.2. NB = No bands.

Isolate	Primer profile $(CAT)_5$	$(CAT)_5$-$(CT)_8$	$(GACA)_4$-$(CGA)_5$	$(GACA)_4$	Haplotype designated
A2	1	1	1	1	1
A17	1	1	1	1	1
A83	2	2	2	2	2
D32	1	1	1	1	1
D42	1	1	1	1	1
B60	1	1	1	1	1
E10	1	1	1	1	1
E51	8	1	1	1	1
E98	1	1	1	1	1
E69	1	1	1	1	1
E81	1	1	1	1	1
F9	2	2	2	2	1
F17	1	1	1	1	1
G2	1	1	1	1	1
G38	1	1	1	1	1
G62	1	1	1	1	1
G75	6	1	1	1	3
G77	1	1	1	1	1
H12	1	1	1	1	1
H15	6	1	1	1	3
H18	1	1	1	1	1
H25	1	1	6	1	4
H29	6	1	10	1	5
H51	3	1	10	1	6
H57	6	1	6	1	5
H74	6	1	6	NB	7
H77	6	1	10	NB	8
H118	8	1	10	NB	8
CRY B	NB	4	NB	NB	-
CRY 0062	NB	1	9	NB	9
P1.19	6	6	8	NB	10
CHA 6	4	5	3	3	11
CHA 9	5	4	4	4	12
CHA 12	4	5	3	3	11
QLD 1	7	3	6	NB	13
QLD 22	9	8	7	5	14
QLD 23	6b	8	6	NB	15
QLD 24	6	7	5	NB	16

The four ISSR primer sets produced a total of 55 scored bands across the 38 isolates. Of these 55 bands, 47 characters were informative and 8 uninformative. Phylogenetic analysis using the Bootstrap method separated the isolates into three main clades. The majority (23 of 28) of the WA *En. eucalypti* population were separated from the remaining isolates (bootstrap support 63%) (Clade 1; Figure 6.3). Although isolates A2, D42 and F17 formed a subclade within Clade 1, bootstrap support for this segregation was low (bootstrap 56%). Two of the WA *En. eucalypti* isolates (F9 and A83), with distinctly different banding patterns, were separated from the remaining WA population as well as the Queensland and South African isolates, with strong bootstrap support (Clade 3; Figure 6.3). The Queensland and South African isolates resided in a single, well-resolved clade along with the *C. cubensis* and unknown *Endothiella* isolates (Clade 2; Figure 6.3). The *Endothiella* isolate collected from *Co. calophylla* (PP1.19) in WA was closely linked to the *C. eucalypti* isolate (QL1) from Queensland, which may indicate that this is the teleomorph of *En. eucalypti*.

Phylogenetic analysis using the Majority Rule method also separated the isolates into three main clades. The majority of the WA isolates (17 isolates) were again separated from the rest of the isolates (Clade 1) (Figure 6.4). Isolates A2, D42 and F17 formed a small subclade (100% bootstrap support) within Clade 1. In 63% of trees generated isolates G75 and H15 were also separated from this clade. Isolate H29 was also separated from Clade 1. Clade 2 comprised of the isolates from Queensland, one from China and South Africa, the *Endothiella* from *Co. calophylla,* the *C. cubensis* isolate as well as WA *En. eucalypti* isolates H57, H74, H77 and H118. Within Clade 2, the *C. cubensis* isolates (Cry B and CH 9) were grouped together and the *C. eucalypti* isolate from Queensland, QL 1 grouped with the isolate from *Co. calophylla* (Figure 6.4). The WA *En. eucalypti* isolates H57, H74, H77 and H118 were separated from the *C. cubensis* and *C. eucalypti* isolates within this clade. *Endothiella eucalypti* isolates A83 and F9 were separated into Clade 3.

Both methods of phylogenetic analysis separate the majority of the WA isolates from the remaining *C. eucalypti* and *C. cubensis* isolates and showed a significant variation within the isolates collected from the Bunbury region. In addition, isolates A83 and F9 are clearly separated from both the *C. eucalypti*, *C. cubensis* and remaining WA isolates in both methods.

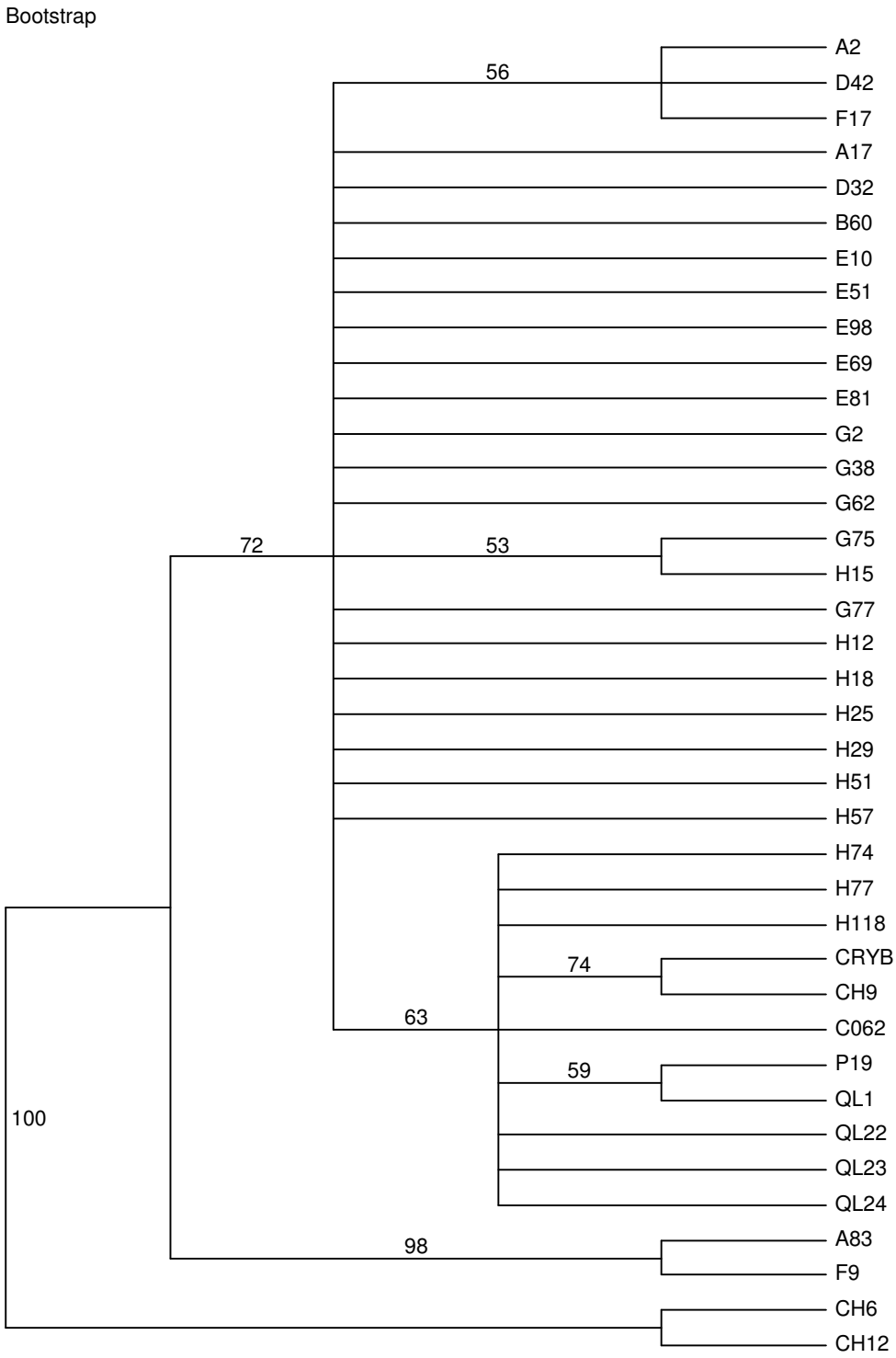


Figure 6.3 Most parsimonious tree generated from ISSR-PCR banding patterns of isolates of *Cryphonectria eucalypti* and its *Endothiella* anamorph collected from Western Australia, Queensland, China and South Africa rooted in *Cryphonectria* isolates CH6 and CH12 From China (see Table 6.1). Bootstrap values are indicated for all the major branches (CI = 0.3793; RI = 0.6604; HI = 0.6207).

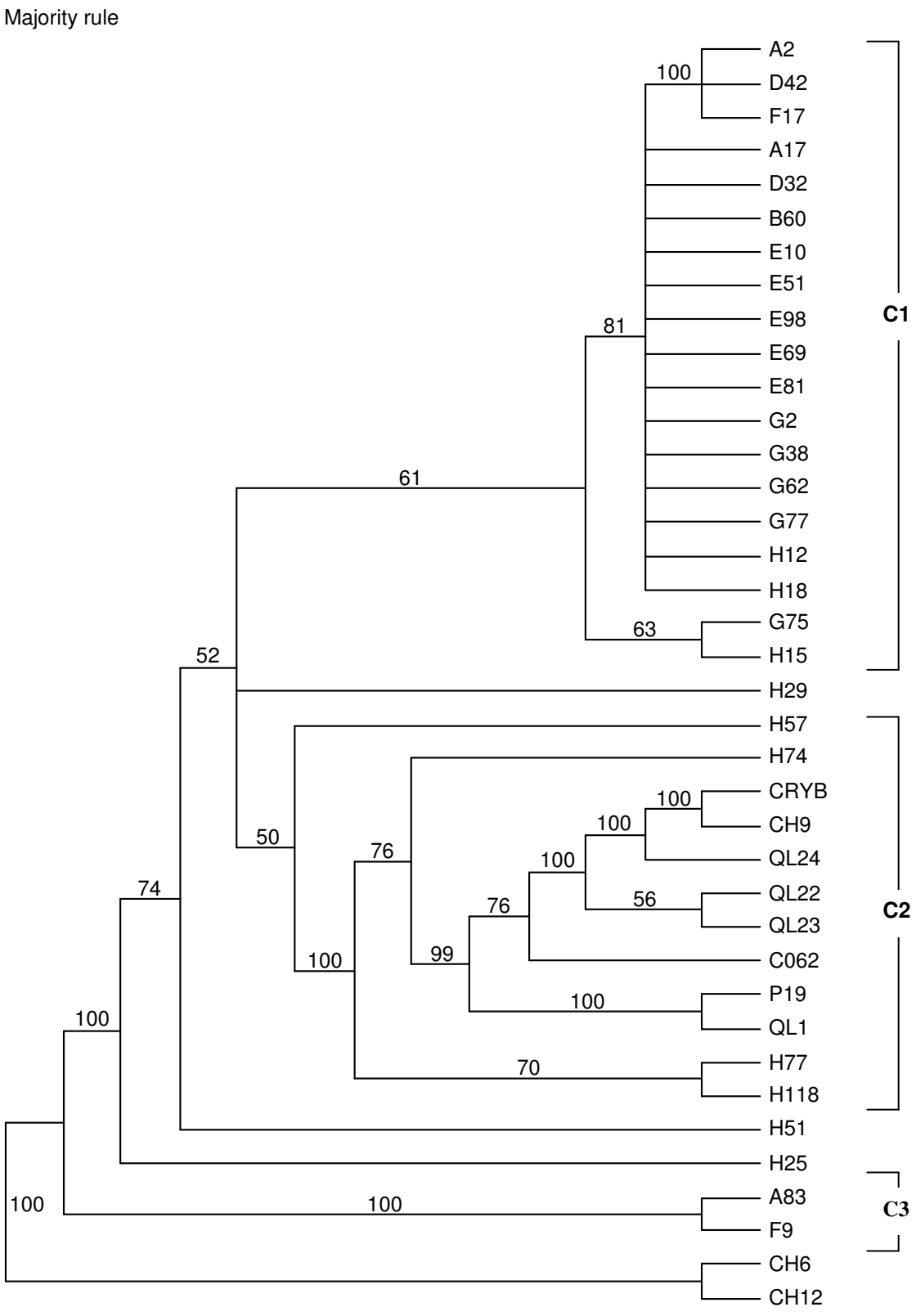


Figure 6.4 Most parsimonious tree generated from banding patterns of isolates of *Cryphonectria eucalypti* and its *Endothiella* anamorph collected from Western Australia, Queensland, China and South Africa rooted in *Cryphonectria* isolates CH6 and CH12 from China. Number of trees which support the isolate grouping are indicated above the branches (CI = 0.53; RI = 0.81; HI = 0.47).

DISCUSSION

Vegetative compatibility and ISSR-PCR identified diversity within the WA population of *En. eucalypti*. The use of ISSR primers was also effective for examining intra- and inter- specific variation within isolates of *Cryphonectria*. However the levels of genotypic diversity calculated differed between VC and ISSR-PCR methods.

Vegetative compatibility assessment of the WA population of *En. eucalypti* indicated an intermediate level (24.14) of genotypic diversity, compared with the levels determined in previous studies which ranged from 1.5 to 38.5 (Burgess *et al.*, 2001; van Heerden and Wingfield, 2001). Twenty-five VC groups were identified from a total sample size of 26 *En. eucalypti* isolates. Paap (2001) reported a similar level of genotypic diversity (22.15) as that recorded in the current study, within a population of *Endothiella* isolated from *Co. calophylla* located in southwestern Australia, with a total of 23 VC groups from a sample size of 24 (Paap, 2001). In comparison, VC studies on a South African population of *C. cubensis* indicated that the population was genetically similar with a genotypic diversity of 9.6 (van Heerden and Wingfield, 2001). Further, a study on isolates of *C. parasitica,* collected from Maryland, represented 31 VC groups in a sample size of 53 and a high genotypic diversity of 49.3 (Liu *et al.*, 1996). The VC method of estimating genotypic diversity was considered by these authors to be a reliable measure that could be used to compare data from different pathosystems with any sample size (van Heerden and Wingfield, 2001).

Previously, the low genotypic diversity within the South African *C. cubensis* population has been attributed to the absence of sexual reproduction (van Heerden and Wingfield, 2001). In contrast, the presence of the sexual state of *C. cubensis* in South America has resulted in high genotypic diversity (van Heerden and Wingfield, 2001). As it is generally accepted that the diversity of an introduced population will be lower than the diversity of an indigenous population, the authors concluded that *C. cubensis* had been present within South America for an extended period of time, whereas it was a recent introduction into South Africa (Van Heerden and Wingfield, 2001). The level of genotypic diversity within the WA population of *En. eucalypti* as established in the current study, may provide evidence for the hypothesis that *En. eucalypti* is part of the indigenous flora associated with WA eucalypts (Davison and Tay, 1983). However, during surveys undertaken on eucalypts within WA (Chapter 2; Paap, 2001; Davison and Coates, 1991), only the *C. eucalypti* anamorph has been observed. If the

teleomorph is not present in WA, this eliminates the possibility of sexual recombination, thus limiting genotypic diversity. However, in the absence of a sexual phase, the level of genotypic diversity within the WA population of *En. eucalypti* may be the result of multiple introductions rather than a long-term evolution, although this seems unlikely. An argument to support the multiple introduction theory exists from a reportedly indigenous population of *Diplodia pinea* from Indonesia that had a significantly lower genotypic diversity than an introduced population in South Africa (Burgess *et al*., 2001). This aberration was attributed to multiple introductions of the pathogen into South Africa thus resulting in a high level of diversity (Burgess *et al*., 2001). A high degree of intraspecific genetic diversity has also been observed in the presumably asexual endophytic fungus, *Phialocephala fortinii* (Grunig *et al*., 2001). It was hypothesised by Grunig *et al.* (2001) that this variation was due to either the presence of a species complex, the presence of an undiscovered sexual state, or by the occurrence of some other kind of recombination such as parasexuality (non-sexual recombination). Given the results of the current study and the presence of the teleomorph on eucalypts in Tasmania and the eastern states (Old *et al.*, 1990; Yuan and Mohammed, 1999; Yuan *et al.*, 1999), it is possible that the teleomorph of *En. eucalypti* exists in WA but has not yet been identified. However, until further surveys (of both native and plantation eucalypts) and population studies are conducted, the origin of *En. eucalypti* remains unclear.

It has been shown that vegetative compatibility of isolates is controlled by allelic interactions. Two strains are incompatible when they have different alleles at one or more vegetatively incompatibility (*vic*) loci (Cortesi and Milgroom, 1998). Therefore, isolates that are vegetatively compatible are considered to be identical at a number of *vic* loci (Cortesi and Milgroom, 1998). For example, the vegetative compatibility of *C. parasitica,* the causal agent of chestnut blight, has been extensively studied and the *vic* loci which determine isolate incompatibility identified (Cortesi and Milgroom, 1998). Although vegetative compatibility has previously been a valuable method for studying diversity of fungi, more detailed analysis is possible where the *vic* genotypes of VC types were known (Cortesi and Milgroom, 1998). This type of more detailed VC analysis may be required when comparing assessments of genotypic variability by conventional and molecular means and may explain the differences in assessments of genotypic diversity between the two methods.

Use of VC analysis to estimate the genotypic diversity within a population is a well established and a widely accepted method (van Heerden and Wingfield, 2001; Burgess *et al.*, 2001; Robin *et al*., 2000; Cortesi and Milgroom, 1998; Cortesi *et al*., 1998). However, in the current study, ISSR-PCR analysis indicated a lower genotypic diversity within the WA *En. eucalypti* population than determined by VC analysis. Previous studies on *D. pinea* have also reported variation in estimates of genotypic diversity between VC and SSR analysis (Burgess *et al.*, in press). In some cases, VC analysis gave a greater genotypic diversity than the molecular technique and in others the reverse was true. The authors indicate that VC groups can have different DNA profiles or the same DNA profile can represent different VC groups. Although the genotypic diversity estimated by ISSR-PCR analysis in the current study may have been lower due to the non-specific nature of the primers, direct comparisons between VC analysis and DNA profiles are not possible. With knowledge of the *vic* loci involved with vegetative compatibility of *En. eucalypti*, (as identified for *C. parasitica*) the accuracy of VC analysis in assessing genotypic diversity will be greatly enhanced. Until then it seems unwise to compare the results of two techniques which assess different aspects of population diversity.

Use of ISSR primers was successful in detecting variation within the WA population of *En. eucalypti* and clearly separated the majority of the WA population from isolates collected in South Africa, Queensland and China. Previous studies with mycorrhizal fungi have reported good inter- and intra- specific separation with microsatellites in comparison with RAPD patterns (Longato and Bonfante, 1997). The low genotypic diversity determined using ISSR-PCR in the current study might therefore be attributed to certain aspects of the methodology, which require refinement. For example, a lack of product amplification, particularly $(GACA)_{4,}$ was recorded for a selection of isolates. This may be the result of a lack of complimentary sites within the template DNA or lack of primer specificity for *En. eucalypti*. Dilution series screening was able to produce a visible product for some isolates, however altering the concentration of DNA did not enhance the results for other isolates, such as the WA *C. cubensis* isolate (Cry B). Development of specific primers for *En. eucalypti* and its teleomorph, together with analysis of a larger sample size would be of great benefit in future population studies of this group of fungi.

Reproducibility is usually assumed when banding patterns of repeated PCR reactions are identical, or when identical banding profiles are obtained regardless of the

concentration of some reaction compounds (Grunig *et al.*, 2001). In the current study a variation in intensity of amplification products with DNA concentration was observed. Similar results have been reported previously (Longato and Bonfante, 1997). Optimal annealing temperature conditions were identified as a major factor contributing to differences in amplification. ISSR primers were evaluated by Blair *et al.* (1999) using a range of annealing temperatures - from 45 °C to 55 °C for AT-rich primers and from 50 °C to 65 °C for GC-rich primers, without modifying the other conditions of the PCR program. As the size of the ISSR primers used in the study were relatively long (27-31 bases), the predicted melting temperatures were 55 °C or higher for the AT-rich primers and 65 °C or higher for all the GC-rich primers. Blair *et al.* (1999) reported that the primers with 75% or greater AT content gave no amplification at either 45 °C or 55 °C annealing temperatures. However, ISSR primers with a greater than one-half GC content produced the same reproducible bands at either 50 °C or 65 °C annealing temperatures (Blair *et al*., 1999). In the current study, although the majority of the isolates (85%) had a distinct, repeatable DNA fingerprint, the remaining isolates either proved difficult to replicate banding patterns or failed to produce a band. The annealing temperature (46 °C) determined as optimal for ensuring clarity of the banding patterns in the current study, is relatively low and may have contributed to a lack of repeatability for some isolates. Therefore, screening a range of *En. eucalypti* isolates with each primer at the annealing temperatures specific to the size and make-up of the primer (AT-rich or GC-rich), may assist in increasing the repeatability of this technique.

For the majority of the *En. eucalypti* isolates, there was no relationship between colony morphology and classification according to VC or ISSR-PCR analysis. The exceptions to this statement are isolates F9 and A83 which separated from the remaining WA *En. eucalypti* isolates in a well resolved clade and shared the same colony morphotypes, which were different from the remaining *En. eucalypti* isolates (Chapter 2). However, there was no relationship between the VC groupings and the pathogenicity of the *En. eucalypti* isolates established in previous chapters (Chapters 3, 4 and 5). This is not unusual as it was concluded previously that VCG and pathogenicity would be correlated coincidentally rather than related to cause-and-effect (Leslie, 1993).

In conclusion, ISSR-PCR and VC analysis were successful in detecting variability within the WA *En. eucalypti* population. With further refinement (such as the development of specific ISSR primers), ISSR-PCR shows potential as a simple, time and cost effective, as well as an accurate method of establishing genotypic diversity

between and within the *En. eucalypti* fungal population. Although VC analysis established variability within the *En. eucalypti* population, it may be argued that this is not a true indication of genotypic diversity as it does not assess variability at a genetic level. It may still be argued that isolates within the same VC group are more genetically similar than those in different VC groups, which can be useful in the identification of limits to gene flow (Couteaudier and Viaud, 1997). However, with the identification and design of species -specific ISSR primers, ISSR-PCR analysis of a larger population size of *En. eucalypti* within WA and comparisons with the eastern states and worldwide shows great potential in investigating the origin of this pathogen with accuracy.

Chapter 7

The role of plant copper status in the susceptibility of *Eucalyptus globulus* to *Endothiella eucalypti*

INTRODUCTION

Copper deficiency in plantation eucalypts has been linked to stem deformities and reduced growth rates, on bauxite mines and ex-pasture sites in southwestern Australia (Gherardi *et al.*, 1999; Dell, 1994; Turnbull *et al.*, 1994). Changes in growth form and rate has also been recorded in response to copper deficiency in the glasshouse (Rogers, 2002; Ishaq, 1999). A survey of two-year-old *Eucalyptus globulus* plantations in southwestern Australia, indicated that 58% of plantations were copper deficient, with copper being the most prevalent nutrient deficiency observed (Rogers, 2002). Stem deformities linked to copper deficiency have also been reported in *Pinus radiata* plantations in southern Australia (Carlyle *et al*., 1989). Copper is thought to play a role in the processes leading to lignification (Dell, 1994). For example, copper-deficient *P. radiata* has lower stem lignin concentrations than non-deficient trees (Carlyle *et al.*, 1989). However, the detailed role of copper in the production of lignin within the plant and the resultant effect on growth form is not yet fully understood (Quartacci *et al.*, 2001; Gherardi *et al.*, 1999).

Activation of the phenylpropanoid pathway and the accumulation of its end products, lignin and phenolics, are important events in incompatible host-pathogen interactions (Cahill *et al.*, 1993). Induced synthesis of phenolics and increased lignification have been associated with resistance against the invasion of fungal pathogens (Cahill *et al.*, 1992; Moerschbacher *et al*., 1988). One hypothesis is that the rapid accumulation of phenolics at the site of infection slows or halts the growth of the pathogen and allows the activation of secondary metabolites which further restrict pathogen invasion (Nicholson and Hammerschmidt, 1992). Phenolic acids and their phenolic precursors are polymerised to lignin by an active mechanism involving free radical intermediates, such as peroxidase (Whetten and Sederoff, 1995; Asiegbu *et al.*, 1994). Increased peroxidase activities have been reported after infection (Moerschbacher *et al*., 1988) or wounding (Asiegbu *et al.*, 1994). Lignin provides a physical barrier to pathogen penetration at the site of infection (Lapierre *et al.*, 2000; Kuc, 1982), whilst many phenolic acids and other phenolic precursors of lignin have strong antifungal activity

(Southerton and Deverall, 1990). For example, Alfenas *et al.* (1982) reported that some of the phenolic compounds derived from eucalypts had a fungitoxic effect on *in vitro* conidial germination of *Cryphonectria cubensis*. The resistance of a host species has been linked to the rate of lignification and accumulations of phenolics (Cahill *et al.*, 1993).

Under conditions of copper deficiency, activity of Cu-dependent enzymes, such as peroxidase, decrease rapidly and an associated decrease in lignification has been reported (Boudet *et al.*, 1995; Dell, 1994). Copper deficiency has been shown to greatly reduce phenolase activity in tomato, sunflower and chrysanthemum (Graves *et al.*, 1979). It has been suggested that the accumulation of phenolic acids and the extent of lignification may be good indicators of plant copper status (Dell, 1994; Graves *et al.*, 1979). Previous studies of *E. globulus* have shown reduced lignification in the stems of plants grown in low copper soils compared to those grown in soils containing adequate copper for plant growth (Ishaq, 1999). In addition, *E. globulus* grown in low copper soils are more susceptible to invasion by *Endothiella* (Ishaq, 1999). However, the results of the study by Ishaq (1999) require further investigation as copper soil status was not controlled during the course of the trial and details on measurements of lignification were not quantitative.

Although copper deficiency has been linked to decreased lignification (Dell, 1994), the mechanisms behind the role of copper in lignification and hence plant resistance to pathogen invasion is still unknown. Therefore, the aim of the current study was to investigate lesion development and to measure the activity of peroxidases and levels of phenolics in *E. globulus* seedlings treated with and without copper, before and after challenge with *En. eucalypti.* It was hypothesised that copper deficient plants will develop larger lesions when inoculated with *En. eucalypti* as a result of reduced activity of defence enzymes and compounds associated with reduced lignin biosynthesis.

MATERIALS AND METHODS

Experimental design

A completely randomised block design consisting of two *E. globulus* provenances (Seed Orchard and Bass Strait) exposed to two copper treatments (no copper (Cu^-) and copper (Cu^+)), two inoculation treatments (+/- pathogen) with six replicate seedlings was undertaken in a glasshouse (Steel and Torrie, 1986). The copper/nutrient treatments were maintained for one year. *Eucalyptus globulus* seedlings were either

inoculated with *En. eucalypti* isolate E81 or a sterile Miracloth® disc as a control. At harvest, three weeks after inoculation, lesions were measured and stem material analysed for soluble protein, peroxidase activity, phenol and copper concentrations.

Biological materials

Seeds of two *E. globulus* provenances (Bass Strait and Seed Orchard, obtained from Integrated Tree Cropping Ltd., Albany WA) were germinated under glasshouse conditions (20-27 °C min-max) in trays of sterilized yellow Karrakatta sand with properties described by Bougher *et al.* (1990). Seedling trays were placed in transparent polyethylene bags (perforated with one 5 mm diameter hole per 100 mm^2 to allow air to circulate) creating a humid environment for seed germination and were watered by hand twice a day with a fine mist from a spray bottle. As the seeds germinated, the bag was gradually removed reducing the humidity of the seedling environment.

After two weeks, the seedlings were transplanted into polyvinyl chloride (PVC) pipe containers as described by Gherardi (1996). Briefly, each PVC pipe (130 mm diameter x 240 mm height) was lined with a polyethylene bag, which was secured at the top of the pipe. Four drainage holes, each 5 mm in diameter, were made in the bottom of the polyethylene bag and the holes covered with a thin layer of polyester fibre filling (Dacron, Tontine) to prevent sand loss. The polyester filling had previously been analysed for copper content by Gherardi (1996) using aqua regia digest and inductively-coupled plasma atomic emission spectrometry (ICPAES) (McGrath and Cunliffe, 1985; In Gherardi, 1996) and no detectable copper was found. Approximately, 4 kg of white washed sand (Soils ain't Soils, Wattlegrove WA; 2.73 mg/kg total copper and 0.016 mg/kg EDTA extractable copper) was placed in each container. Each container was watered to container capacity with double de-ionized water (DDI) daily for seven days prior to planting. DDI water was obtained by passing water through a reverse osmosis water purifier (consisting of 1 carbon cartridge and 2 De-ionizing resin cartridges) (Unicorn Water Purification, Australia).

As the seedlings were placed into the PVC containers, a piece of polyester fibre filling was placed on the sand surface to prevent soil disturbance during watering until the seedlings were established. Each container was given 100 mL DDI water daily for two weeks until nutrient treatments were established (Figure 7.1). The experiment was conducted in a evaporatively-cooled glasshouse in early spring (September).

The *En. eucalypti* isolate E81 was passaged through a *E. globulus* host prior to use as described previously (Chapter 3). The isolate was maintained on 1/2PDA at 20 °C under continuous near blue light. Actively growing colonies of the isolate were subcultured onto 1/2PDA plates containing sterile Miracloth® (Calbiochem, Victoria, Australia) discs and allowed to grow for seven days as described in Chapter 3.

Figure 7.1 Four-week-old *Eucalyptus globulus* seedlings becoming established in sand-filled pipe containers prior to commencement of nutrient treatments.

Nutrient solutions

Nutrients and their concentrations were as described by Gherardi (1996) and Rogers (2002) (Table 7.1). Macronutrient stock solutions were purified to remove traces of heavy metals, including copper, by complexing with dithizone in chloroform (Hewitt, 1966). Micronutrient solutions were made from the purest form available as they could not be purified for copper as this would remove other essential micronutrient cations.

Basal nutrients were added to the seedlings every second day (100 mL to each container) at half strength for the first two months and thereafter at full strength when plants had grown new leaves (Table 7.1). Containers were flushed beyond field capacity every second day with DDI to prevent nutrient accumulation. Throughout this chapter, plants treated with the nutrient solution containing copper are referred to as Cu^{+} plants and those treated with the nutrient solution without copper as Cu^{-}.

Table 7.1 Composition of basal nutrient solution and copper used to grow *Eucalyptus globulus* seedlings.

Nutrient Salt	Working Solution [µM]
$Ca(NO_3)_2.4H_2O$	500
NH_4NO_3	500
KNO_3	300
$MgSO_4.7H_2O$	300
KH_2PO_4	40
$K_2HPO_4.3H_2O$	40
H_3BO_3	10
$MnSO_4.H_20$	3
$ZnSO_4.7H_2O$	1
$CoSO_4.7H_20$	0.05
$NaMoO_4.2H_20$	0.025
$CuSO_4.5H_2O$ (in Cu^+ treatment solutions)	10

Inoculation and harvest

Using a sterile single edged blade, a leaf pair was removed from the main stem of each *E. globulus* seedling approximately 20 cm above the base of the stem. At this stage, the plants were one-year-old and 1 m tall. The leaves were placed in paper bags and oven dried at 60 °C for two weeks for copper analysis. Where the leaves had been removed, a 5 mm diameter Miracloth® disc colonised with the actively growing seven-day-old *En. eucalypti* isolate was secured to the trunk (mycelia side against the trunk covering the severed petioles) with Parafilm and flagging tape. This method of inoculation was chosen in preference to the underbark method as it caused the least damage to stem tissue, which may have altered the host defence responses. Non-colonised sterile discs were used as controls and secured to the trunk in the same manner as the colonised discs.

Twenty-one days after inoculation, the plants were harvested at the base of the stem and stored on ice. Lesion extension was measured and the stem divided into four x 10 mm segments. The first segment containing the last 10 mm of the lesion (if present) and the three remaining 10 mm segments were harvested up the stem (Figure 7.2). If a lesion was not present (control seedlings), the stem was divided into four segments with the first 10 mm segment commencing at the point of inoculation . Each of the four segments were then cut longitudinally and placed in a 1.5 mL microfuge tube, frozen in liquid nitrogen and stored at -80 °C for biochemical analysis.

Once the stem sections were removed from the plant, a 100 mm section of the stem (above the harvested area) (Figure 7.2) and the top youngest fully expanded leaf pair (YFEL) were placed in separate paper bags and oven dried at 60 °C for two weeks for copper analysis.

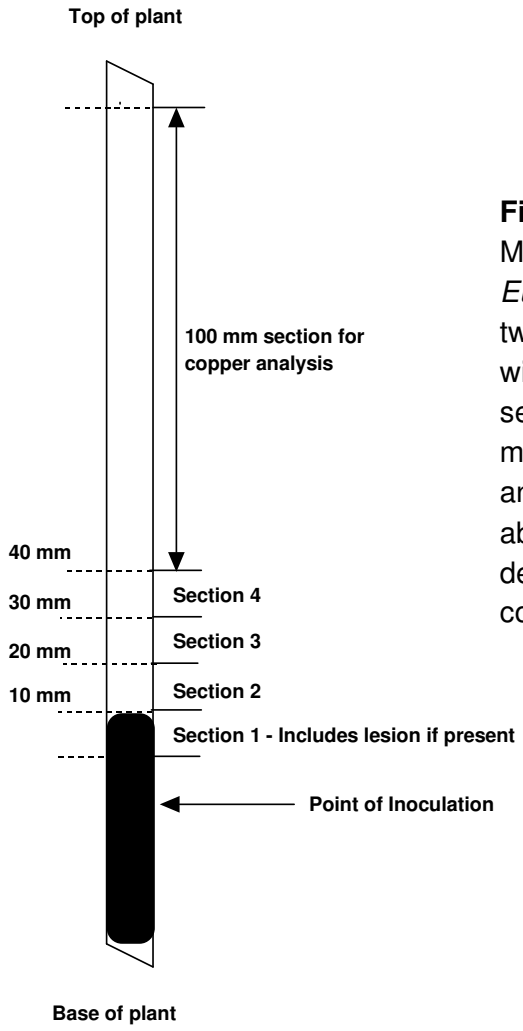


Figure 7.2
Method for harvesting *Eucalyptus globulus* stems twenty-one days after inoculation with *Endothiella eucalypti*. Stem sections 1 to 4 were placed into microfuge tubes for biochemical analysis. The 100 mm section above section 4 was used for the determination of stem copper concentration.

Copper analysis

The leaf and stem samples were finely ground in a stainless steel coffee grinder (Breville Holdings Pty Ltd, Australia), digested in nitric acid (HNO_3) according to Gheradi (1996) and analysed by ICPAES as described by Zarcinas *et al.* (1987). Samples (approximately 500 g) were predigested overnight in concentrated HNO_3 (3 mL) before being placed in an aluminum digestion block and the temperature slowly increased to 140 °C over 30 to 40 minutes until the liquid volume in each tube was reduced to less than 1 mL. A further 2 mL HNO_3 was then added to each tube and left to continue digesting at 140 °C. When the liquid in all tubes was reduced to below 1 mL, the tubes were taken out of the digestion blocks, allowed to cool and the liquid

contents diluted to a 5 mL volume with 1% HNO_3 (w/w). Eucalypt standard reference material obtained from the State Chemistry Laboratory, Victoria, was digested with each batch of samples in order to monitor the digestion method for element recovery and ICPAES accuracy. Copper levels were expressed as mg/g dry weight of plant material. Plants were classed as deficient if foliar copper concentrations were within the deficient concentration range (0.6 - 1.6 mg kg^{-1} dry wt) defined by Dell *et al.* (1995).

Enzyme extraction and assays

Stem material was powdered using liquid nitrogen in a mortar and pestle and extracted in 50 mM phosphate extraction buffer (pH 7). The extract was centrifuged at 14 000 *g* for 10 min and the supernatant removed for analysis of soluble peroxidase (25 µL), soluble phenolics (15 µL) and soluble protein (25 µL) content. The remaining pellet was washed twice with 5 mM phosphate buffer (pH 7) then 5 mM phosphate buffer containing Tween 80 (polyoxyethylenesorbitan monooleate: Sigma-Aldrich Pty. Ltd.) was added and the mixture vortexed and centrifuged at 14 000 *g* for 1 min. The supernatant was removed and static peroxidase (PO) activity determined (see details below). The pellet was washed as before with 5 mM phosphate buffer before leaving the pellet suspended in phosphate buffer containing 1M NaCl at 4 °C for 12 hours. After this time, the mixture was centrifuged at 14 000 *g* for 5 min and the supernatant removed to determine ionically bound PO activity. The remaining pellet was washed twice in 5 mM phosphate buffer and re-suspended in fresh buffer. One tenth of the pellet suspended in 5 mM phosphate buffer was used to determine covalent PO activity.

Protein content was determined using a Protein Determination Kit (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547), based on the method of Bradford (1976), and was expressed as mg ml^{-1} by comparing the absorbance at 595 nm with that of a bovine serum albumin (BSA) standard curve. The level of soluble phenolics was determined using Folin and Ciocalteu's Phenol reagent (Sigma Chemical Company, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) and expressed as µmol mg^{-1} protein by comparing absorbance at 725 nm with that of a 4-coumarate standard curve according to Lowry (1951).

Peroxidase assays

Peroxidase fractionation was undertaken according to Sato *et al.* (1993). Aliquots of 25, 50 and 100 µL of the extracts were used to determine soluble, static and ionic PO assays, respectively. The aliquots were made up to 2 mL with 50 mM phosphate/citrate buffer (pH 5.8) containing 20 mM guaiacol (Sigma Chemical Company, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). The reaction mixture was incubated at 30 °C for 1 min before the reaction was started with the addition of 50 µL of 0.6 M H_2O_2. The absorbance of the solution was then measured at a wavelength of 470 nm, 10 min after the addition of the H_2O_2. PO activity was expressed as pkat mg^{-1} protein using the extinction coefficient of tetraguaiacol (2.66 x $10^7 cm^2 mol^{-1}$).

Covalently bound PO was determined by removing one tenth of the pellet, adding 800 µL of the 50 mM phosphate/citrate buffer containing guaiacol and incubating the solution for 1 min at 30 °C. The reaction was initiated with the addition of 50 µL of 0.6 M H_2O_2 and stopped after 5 minutes by centrifugation. The absorbance of the solution was determined immediately following centrifugation at 470 nm and expressed as defined previously.

Statistical analysis

Data obtained for lesion extension, phenolic and peroxidase assays and copper were analysed separately by ANOVA using Statistica Version 4.1 (StatSoft® Inc., OK, USA). Data were assessed for homogeneity, variation of the mean from the variance and fit to a normal distribution. Means were compared by LSD ($p \leq 0.01$) and presented with standard error of the mean.

RESULTS

Effect of copper

At the time of inoculation, the Cu^- seedlings had typical copper deficient symptoms, such as twisted stems (near top of plant; Figure 7.3A), stunted and disfigured young shoots. Cu^+ seedlings were often taller than the Cu^- seedlings with healthy, well formed, young shoots (Figure 7.3B). The Cu^+ *E. globulus* seedlings had significantly ($p \leq 0.0001$) higher tissue copper concentrations compared to Cu^- seedlings (Table 7.2a).

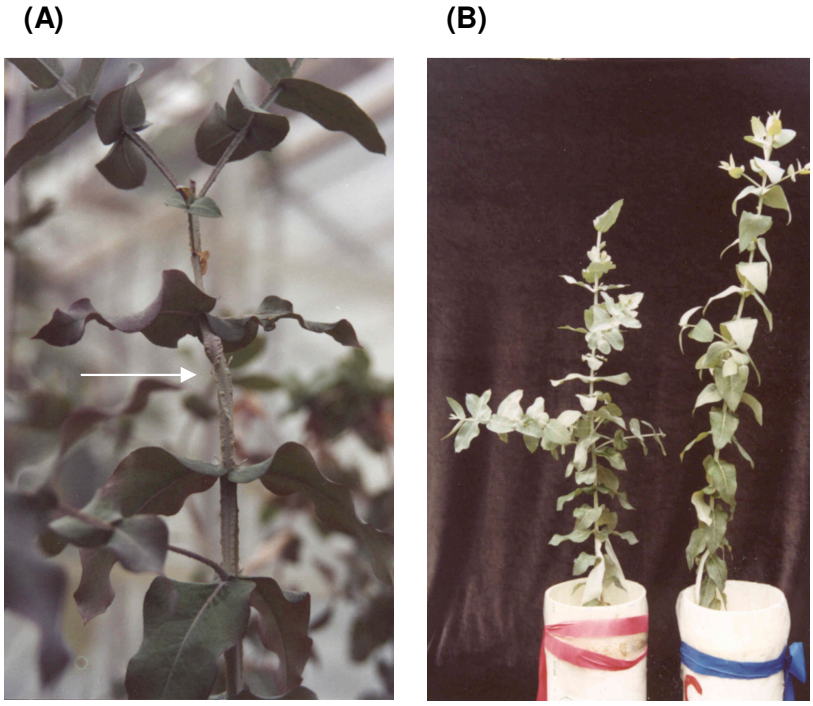


Figure 7.3 Seedlings of *Eucalyptus globulus* which did not have copper in the nutrient treatments often had twisted stems (A) and were generally smaller than seedlings which received copper (B) (copper treated seedling on right).

Table 7.2 ANOVA of tissue copper levels (µg/g dry wt) in the leaves and stems of Seed Orchard and Bass Strait *Eucalyptus globulus* seedlings, prior to inoculation with *En. eucalypti* and at the time of trial harvest. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 3, 28)	P
Provenance (1)	1.364	0.274
Copper (2)	133.136	**0.000**
1 x 2	1.365	0.274

(b)

Effect	Leaves prior to inoculation	Leaves at harvest	Stems at harvest
Copper	**p=0.000**	**p=0.000**	**p=0.000**

Copper levels in leaves prior to inoculation

Prior to inoculation, the leaves of Cu^+ seedlings had significantly ($p< 0.0001$) higher copper levels than Cu^- seedlings (Table 7.2b; Figure 7.4A). There was no difference ($p=0.274$) in the copper concentrations of leaves between provenances (Table 7.2a). However, overall the Seed Orchard seedlings had higher levels of copper compared with the Bass Strait seedlings (Figure 7.4A).

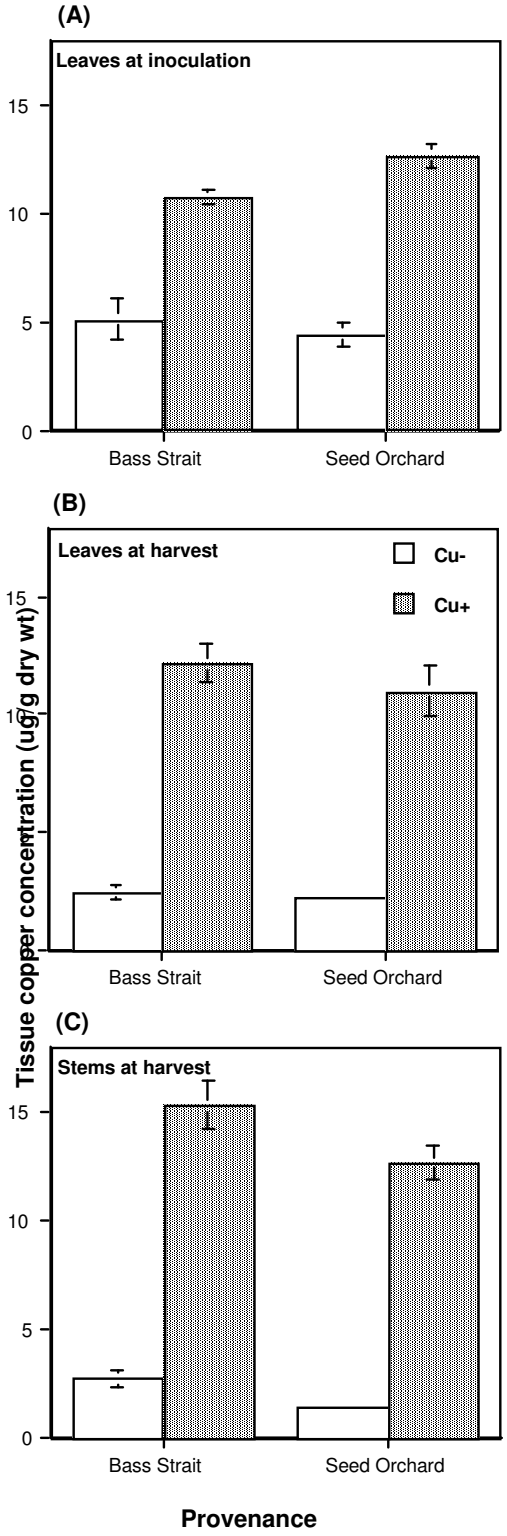


Figure 7.4
Tissue copper levels (µg/g dry wt) in leaves and stems of *Eucalyptus globulus* seedlings of Bass Strait and Seed Orchard provenances before inoculation with *Endothiella eucalypti* (A) and at harvest (B and C). Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal.

Copper levels in leaves and stems at harvest

Inoculation with *En. eucalypti* did not significantly (p=0.06 and p=0.15, respectively) affect the levels of copper in the leaves and stems at harvest; therefore data for inoculated and non-inoculated material were analysed together. The copper levels in

the leaves and stems at harvest were significantly ($p<0.0001$) greater in the Cu^+ seedlings than the Cu^- seedlings for both provenances (Figure 7.4B and C; Table 7.2b). Copper levels in the leaves and stems of the Cu^+ Bass Strait provenance were higher compared to the Cu^+ Seed Orchard provenance (Figure 7.4B and C). At harvest, the stems of the Cu^+ seedlings contained higher copper levels compared to the leaves of Cu^+ seedlings (Figure 7.4B and C).

There was a correlation ($r=0.75$) between leaf copper concentration at inoculation and at harvest (data not shown). In addition, there was a strong correlation ($r=0.82$) between copper levels in the leaves and stems of the seedlings at harvest (data not shown) indicating that either tissue type may be used for determining whole plant copper status.

Lesion development

There was no significant ($p = 0.315$; Table 7.3) difference in lesion development between Cu^- or Cu^+ seedlings inoculated with *En. eucalypti* (Figure 7.5). Although not significant ($p=0.122$) (Table 7.3), Bass Strait seedlings had larger lesions than the Seed Orchard seedlings (Figure 7.5). There was no correlation between lesion extension and copper levels in the leaves ($r=0.05$) or stems ($r=0.12$) at harvest (data not shown). Lesions often girdled the stems of *E. globulus* (Figure 7.6A) and were associated with wilting of seedling crowns regardless of provenance (Figure 7.6B).

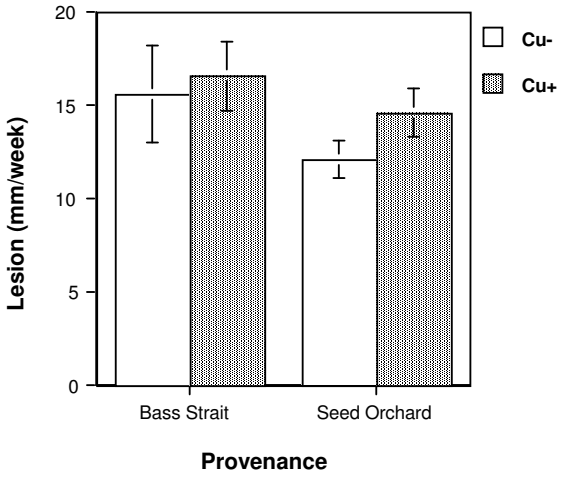


Figure 7.5 Lesion extension (mm/week) in the stems of Cu^+ and Cu^- *Eucalyptus globulus* seedlings of Bass Strait and Seed Orchard provenance before and after inoculation with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

Table 7.3 ANOVA of lesion extension (mm/week) in stems of Cu^+ and Cu^- Bass Strait and Seed Orchard *Eucalyptus globulus* provenances caused by inoculation with *Endothiella eucalypti* as shown in Figure 7.5.

Effect	MS Effect	MS Error	F (df 1, 18)	P
Copper (1)	16.535	15.456	1.070	0.315
Provenance (2)	40.792	15.456	2.639	0.122
1 x 2	3.04811	15.45573	0.197	0.662

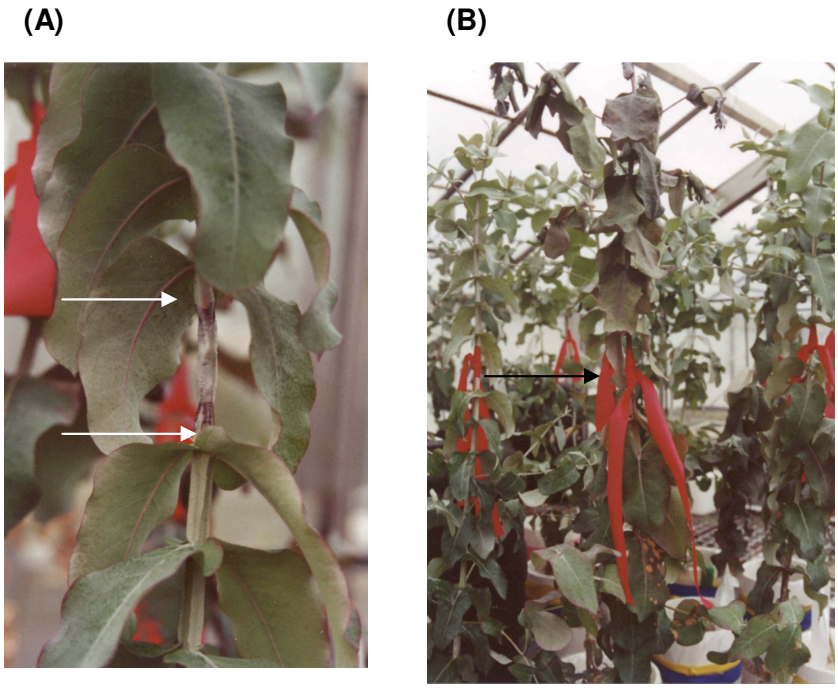


Figure 7.6 Examples of lesion development (A) (arrows indicate extent of lesion) and girdling of stems (arrow indicates point of inoculation) leading to wilting of the crown of *Eucalyptus globulus* (B) inoculated with *Endothiella eucalypti*.

Host defence enzyme activity

Of the four segments examined, changes in PO activity and phenolic accumulation were more pronounced in the first and second stem segments (Appendix Tables 7.1 to 7.5). Therefore, data discussed below relate to ANOVA of stem segments 1 and 2 only, unless specified.

Effect of copper

Non-inoculated Cu^+ seedlings had higher levels of soluble and bound phenolics compared to Cu^- seedlings, although this trend was not significant (p=0.945 and p=0.507, respectively) (Tables 7.4(a) and 7.5(a); Figure 7.7). Ionic PO activity was higher throughout the stems of Cu^+ plants than Cu^- plants (Figures 7.8C and 7.9C). Soluble PO (throughout the stem) and covalent PO (especially in segments 2 and 3)

activity was higher in Cu^+ Seed Orchard seedlings compared to Cu^- Seed Orchard seedlings (Figure 7.9A and D). Seed orchard seedlings had significantly (p=0.005 and p=0.003, respectively) higher levels of soluble and bound phenolics in segment 1 (Tables 7.4(b) and 7.5(b)).

Table 7.4 ANOVA of soluble phenolics (mM mg protein^{-1}) for stem segments one and two of *Eucalyptus globulus* of Bass Strait and Seed Orchard provenances with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 2, 13)	P
Copper (1)	0.057	0.945
Provenance (2)	6.806	**0.009**
1 x 2	0.057	0.944

(b)

Effect	Segment 1	Segment 2
Provenance	**p=0.005**	p=0.032

Table 7.5 ANOVA of bound phenolics (mM mg protein^{-1}) for stem segments one and two of *Eucalyptus globulus* of Bass Strait and Seed Orchard provenances with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 2, 13)	P
Copper	0.716	0.507
Provenance (2)	8.250	**0.005**
1 x 2	1.209	0.330

(b)

Effect	Segment 1	Segment 2
Provenance	**p=0.003**	p=0.282

Effect of inoculation in Cu^- seedlings

Soluble and bound phenolic levels were higher throughout the stems of inoculated compared to non-inoculated, Cu^- seedlings (Figure 7.7). Levels of soluble phenolics were significantly (p=0.0004) higher in the Cu^- Seed Orchard seedlings after inoculation, compared to Cu^- Bass Strait seedlings (Table 7.6(a)), especially in segment one (p=0.003) (Table 7.6(b)). Activity of soluble (Bass Strait seedlings only), static and ionic PO increased markedly in segment one of *E. globulus* stems following inoculation (Figures 7.8 and 7.9). Covalent PO activity increased throughout the stem of the Bass Strait seedlings following inoculation (Figure 7.8D).

Table 7.6 ANOVA of soluble phenolics (mM mg protein^{-1}) for all stem segments of Cu^{-} *Eucalyptus globulus* of Bass Strait and Seed Orchard provenances. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 4, 15)	P
Provenance (1)	1.732	**0.0004**
Inoculation (2)	1.987	0.148
1 x 2	0.614	0.659

(b)

Effect	Segment 1	Segment 2	Segment 3	Segment 4
Provenance	**p=0.003**	p=0.020	**0.002**	0.0432

Effect of copper and provenance

Seed Orchard seedlings had significantly (p=0.005 and p=0.003, respectively) higher levels of soluble phenolics (throughout the stem) and bound phenolics (in segments 1 and 2) compared to Bass Strait seedlings (Tables 7.7(a) and 7.8(a); Figure 7.7). PO activity was higher (although not significant at p≤0.01) in Seed Orchard seedlings compared with Bass Strait seedlings (Figures 7.8 and 7.9).

Table 7.7 ANOVA of soluble phenolics (mM mg protein^{-1}) for all four stem segments of Bass Strait and Seed Orchard *Eucalyptus globulus* provenances, with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 4, 32)	P
Copper (1)	0.64	0.63
Provenance (2)	4.55	**0.005**
Inoculation (3)	4.92	**0.003**
1 x 2	0.78	0.55
1 x 3	0.41	0.79
2 x 3	1.43	0.24
1 x 2 x 3	0.56	0.69

(b)

Effect	Segment 1	Segment 2	Segment 3	Segment 4
Provenance	**p=0.003**	**p=0.030**	**p=0.008**	**p=0.020**
Inoculation	**p=0.088**	**p=0.012**	p=0.140	p=0.38

Table 7.8 ANOVA of bound phenolics (mM mg protein^{-1}) for stem segments one and two of Bass Strait and Seed Orchard *Eucalyptus globulus* provenances, with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 2, 34)	P
Copper (1)	0.241	0.787
Provenance (2)	6.821	**0.003**
Inoculation (3)	5.919	**0.006**
1 x 2	0.274	0.782
1 x 3	0.317	0.730
2 x 3	0.656	0.525
1 x 2 x 3	0.737	0.486

(b)

Effect	Segment 1	Segment 2
Provenance	**p=0.0007**	**p=0.039**
Inoculation	**p=0.001**	**p=0.011**

Table 7.9 ANOVA of bound phenolics (mM mg protein^{-1}) for all four stem segments of Bass Strait and Seed Orchard *Eucalyptus globulus* provenances, with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 4, 32)	P
Copper (1)	0.114	0.97
Provenance (2)	3.27	0.023
Inoculation (3)	4.66	**0.004**
1 x 2	0.18	0.95
1 x 3	0.22	0.92
2 x 3	1.37	0.83
1 x 2 x 3	0.89	0.48

(b)

Effect	Segment 1	Segment 2	Segment 3	Segment 4
Inoculation	**p=0.001**	**p=0.011**	**p=0.036**	p=0.50

Effect of copper and inoculation

The levels of soluble and bound phenolics increased significantly (p=0.003 and p=0.004, respectively) throughout the *E. globulus* stems following inoculation (Tables 7.7(a) and 7.9(a)). Activity of static and ionic PO in stem segments 1 and 2 was significantly (p=0.001 and p=0.008, respectively) greater in inoculated compared with non-inoculated stems in both *E. globulus* provenances (Tables 7.10(a) and 7.11(a)). This significant (p<0.001 and p=0.002) increase in the activity of static and ionic PO, respectively, was especially evident in segment one (Tables 7.10(b) and 7.11(b)).

Table 7.10 ANOVA of static peroxidase (nkat mg protein^{-1}) for stem segments one and two of Bass Strait and Seed Orchard *Eucalyptus globulus* provenances, with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

Effect	Rao's R (df 2, 33)	P
Copper (1)	0.72	0.49
Provenance (2)	1.25	0.30
Inoculation (3)	8.07	**0.001**
1 x 2	0.16	0.85
1 x 3	0.34	0.71
2 x 3	2.44	0.10
1 x 2 x 3	0.30	0.74

(b)

Effect	Segment 1	Segment 2
Inoculation	**p<0.001**	**p=0.016**

Table 7.11 ANOVA of ionic peroxidase (pkat mg protein^{-1}) for stem segments one and two of Bass Strait and Seed Orchard *Eucalyptus globulus* provenances, with or without copper treatment. (a) Initial ANOVA. (b) Univariate tests where the multivariate effect or interaction is significant. Significant values are in bold font.

(a)

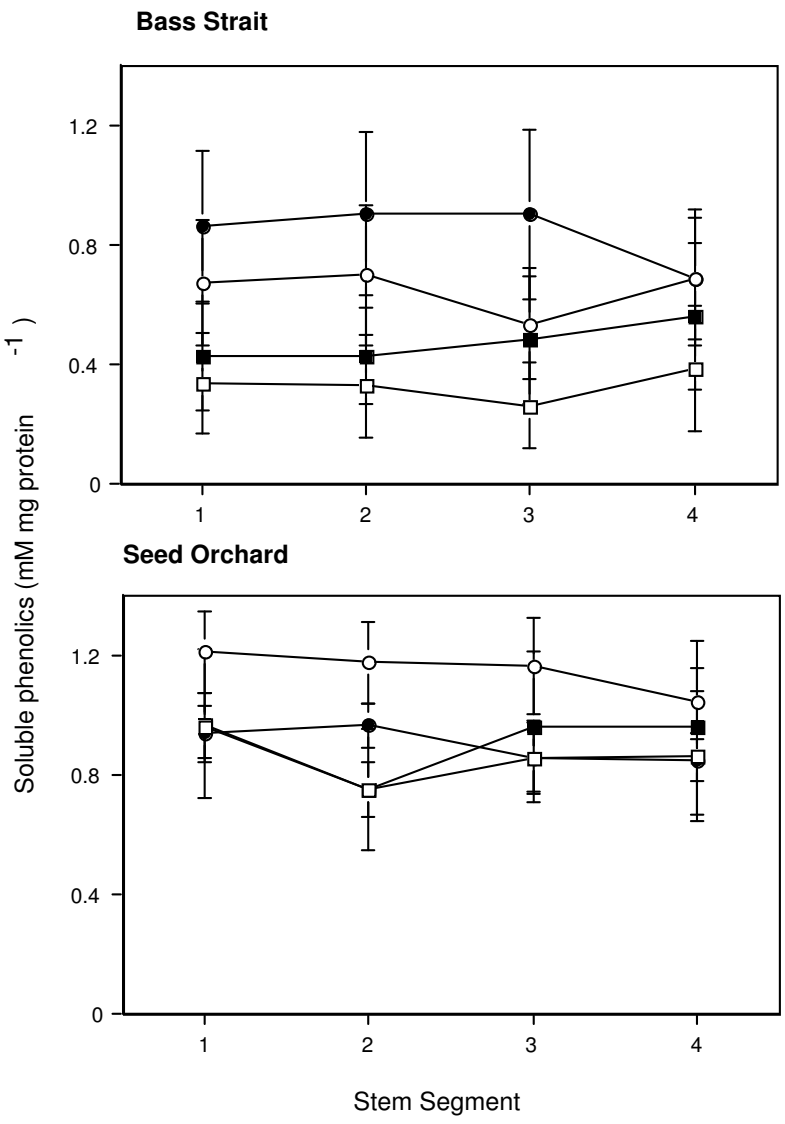
Effect	Rao's R (df 2, 33)	P
Copper (1)	0.69	0.50
Provenance (2)	4.12	0.026
Inoculation (3)	5.75	**0.008**
1 x 2	0.19	0.83
1 x 3	2.19	0.13
2 x 3	2.16	0.13
1 x 2 x 3	0.27	0.77

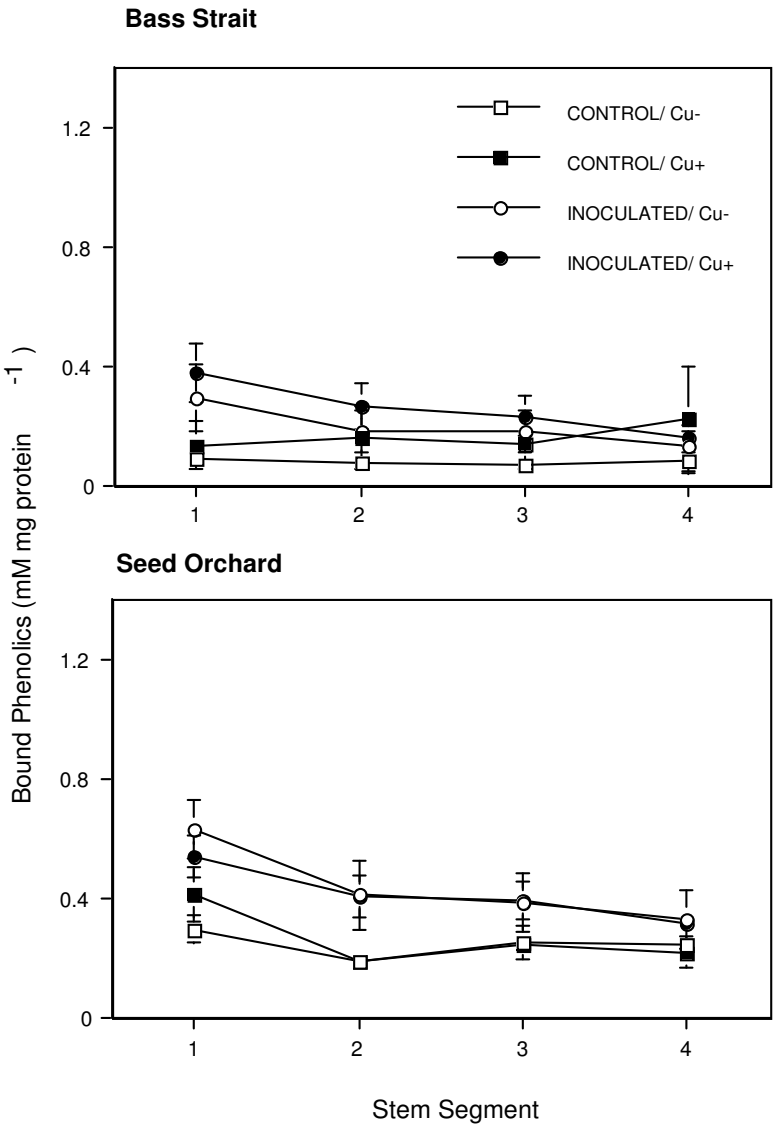
(b)

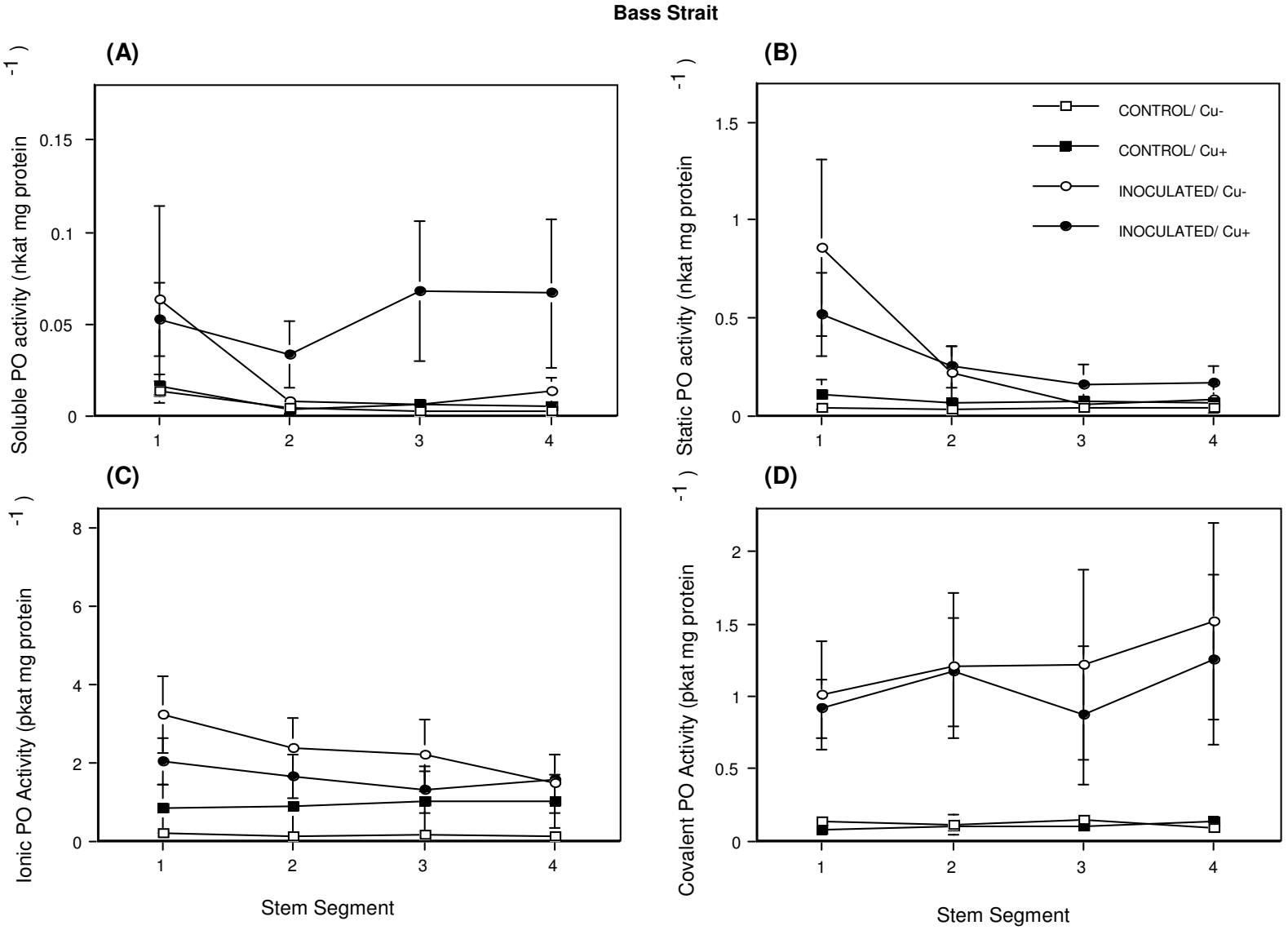
Effect	Segment 1	Segment 2
Inoculation	**p=0.002**	**p=0.02**

Cu^+ Bass Strait seedlings had higher levels of soluble and bound phenolics after inoculation than Cu^- Bass Strait seedlings, although not significantly (at $p \leq 0.01$) (Figure 7.7). Inoculation increased the levels of soluble phenolics in the stems of Cu^+ Bass Strait seedlings (Figure 7.7). Levels of soluble phenolics in the stems of Cu^+ Seed Orchard seedlings increased only in segment 2 following inoculation compared to non-inoculated Cu^+ seedlings (Figure 7.7). Inoculation of Cu^+ Seed Orchard seedlings resulted in a decrease in the levels of soluble PO in segment 1 of the stem to below control levels. In contrast, inoculation of Cu^+ Bass Strait seedlings resulted in an increase in soluble PO activity throughout the stem. The activity of ionic PO increased throughout the stems of Cu^+ seedlings following inoculation, however the levels were

not as high as those recorded in the stems of Cu^- seedlings following inoculation (Figures 7.8 and 7.9). A similar trend was observed in covalent PO activity in the stems of Bass Strait seedlings (Figure 7.8). In contrast, the activity of covalent PO decreased throughout the stems of Cu^+ Seed Orchard seedlings compared to Cu^- seedlings following inoculation (Figure 7.9).


Bass Strait
Seed Orchard
Soluble phenolics (mM mg protein -1)
1.2
0.8
0.4
0
1
2
3
4
Stem Segment


Bass Strait
Seed Orchard
Bound Phenolics (mM mg protein -1)
CONTROL/ Cu-
CONTROL/ Cu+
INOCULATED/ Cu-
INOCULATED/ Cu+
1.2
0.8
0.4
0
1
2
3
4
Stem Segment


Bass Strait
(A)
(B)
(C)
(D)
Soluble PO activity (nkat mg protein -1)
Static PO activity (nkat mg protein -1)
Ionic PO Activity (pkat mg protein -1)
Covalent PO Activity (pkat mg protein -1)
CONTROL/ Cu-
CONTROL/ Cu+
INOCULATED/ Cu-
INOCULATED/ Cu+
Stem Segment
Stem Segment

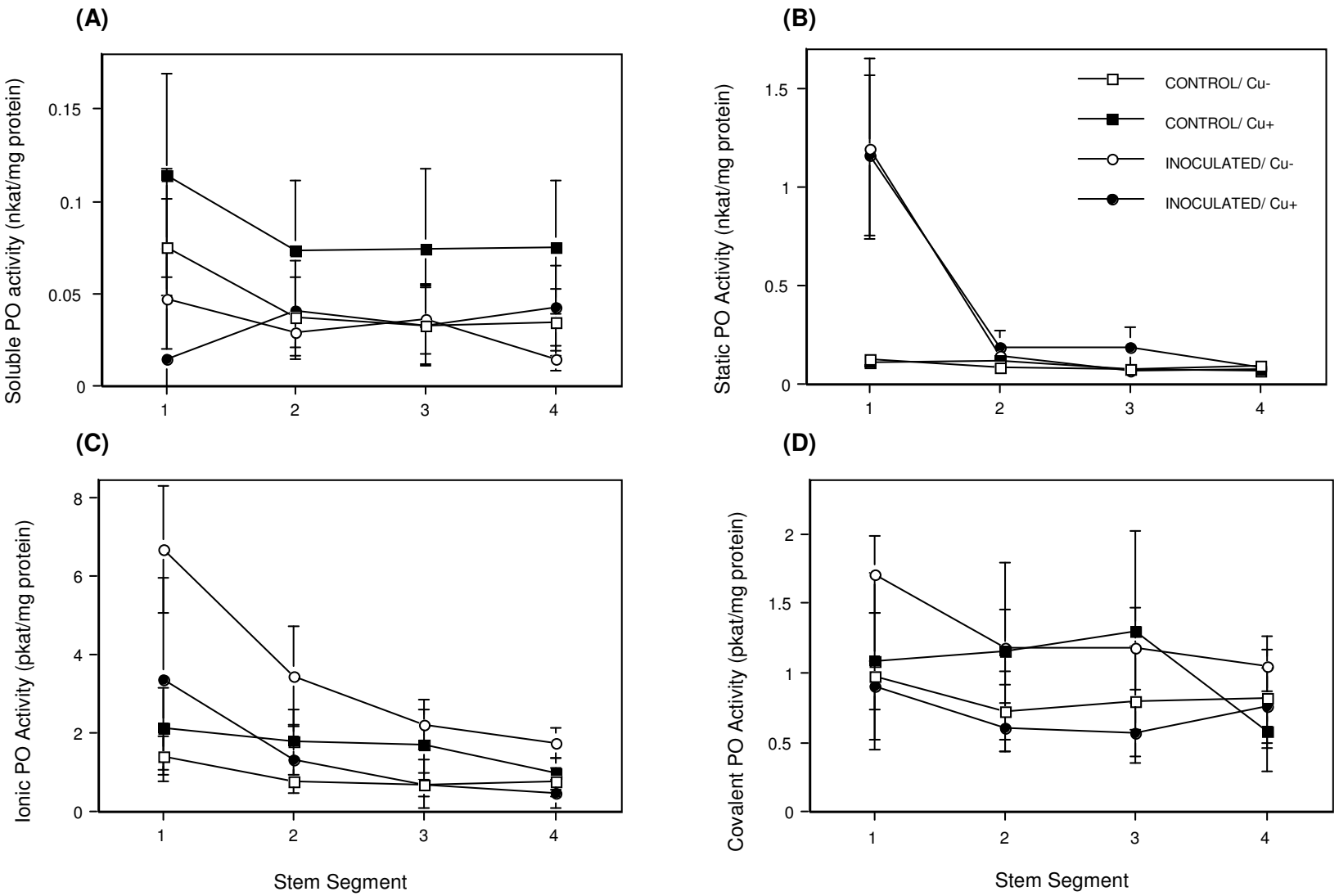

Seed Orchard
(A)
Soluble PO activity (nkat/mg protein)
0
0.05
0.1
0.15
1
2
3
4
(B)
Static PO Activity (nkat/mg protein)
0
0.5
1
1.5
CONTROL/ Cu-
CONTROL/ Cu+
INOCULATED/ Cu-
INOCULATED/ Cu+
(C)
Ionic PO Activity (pkat/mg protein)
0
2
4
6
8
Stem Segment
(D)
Covalent PO Activity (pkat/mg protein)
0
0.5
1
1.5
2
Stem Segment

Figure 7.7 Levels of soluble and bound phenolics (mM mg protein^{-1}) in stems of Cu^{+} and Cu^{-} *Eucalyptus globulus* of Bass Strait and Seed Orchard provenances, before and after inoculation with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

Figure 7.8 Levels of (A) soluble (nkat mg protein^{-1}), (B) static (nkat mg protein^{-1}), (C) ionic (pkat mg protein^{-1}) and (D) covalent (pkat mg protein^{-1}) peroxidase in stems of Cu^{+} and Cu^{-} *Eucalyptus globulus* of Bass Strait provenance, before and after inoculation with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

Figure 7.9 Levels of (A) soluble (nkat mg protein^{-1}), (B) static (nkat mg protein^{-1}), (C) ionic (pkat mg protein^{-1}) and(D) covalent (pkat mg protein^{-1}) peroxidase in stems of Cu^{+} and Cu^{-} *Eucalyptus globulus* of Seed Orchard provenance, before and after inoculation with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

DISCUSSION

Eucalyptus globulus seedlings grown in the presence of copper (Cu^+) were no more resistant to *En. eucalypti* colonisation than seedlings grown without copper (Cu^-). Although the seedlings grown in the absence of copper exhibited typical symptoms of copper deficiency, such as twisted leaves and stems and stunted growth, lesion development was similar to copper adequate seedlings. The increase in phenolic accumulation and PO activity which was observed in response to inoculation with *En. eucalypti* varied according to *E. globulus* provenance rather than plant copper status.

Under copper deficient conditions, the differences in host defence activity between the two *E. globulus* provenances was marked. The Seed Orchard seedlings had higher constitutive levels of peroxidases and phenolics compared to the Bass Strait *E. globulus* seedlings. Higher constitutive levels of defence-related compounds have previously been associated with more resistant plant varieties (Cahill *et al*., 1993). It is interesting to note that the Seed Orchard seedlings developed smaller lesions and had lower levels of copper in the stems at harvest compared to the Bass Strait seedlings, although these differences were not statistically significant. The results of the current study indicate that the Seed Orchard provenance may be a more resistant variety of *E. globulus* to *En. eucalypti* compared to the Bass Strait seedlings. Further studies examining constitutive levels of key enzymes/compounds involved in the host defence response of *E. globulus* provenances which have been shown to be resistant and susceptible to disease are required to determine whether more resistant *E. globulus* provenances have higher constitutive levels of defence compounds than susceptible provenances. If the levels of the constitutive host defence enzymes are associated with the overall resistance of *E. globulus* to *En. eucalypti*, this may be a good method of screening for more resistant *E. globulus* provenances.

In the current study, external copper concentration did not affect the extent of lesion development in either of the *E. globulus* provenances. Previously, Ishaq (1999) investigated the impact of external copper concentration (5 mg Cu/ plant compared to nil Cu/ plant) on the susceptibility of twenty-five *E. globulus* families to *Endothiella* in the glasshouse. The results of this study showed variation in the susceptibility of the *E. globulus* families to *Endothiella*, however plants with low stem copper concentrations developed larger lesions than plants with higher levels of copper in plant

tissue. One *E. globulus* family did not follow this trend and recorded larger lesions when grown under adequate copper conditions compared to copper deficient conditions (Ishaq, 1999). It is hypothesised that the variation in the susceptibility of *E. globulus* to *En. eucalypti* under different external copper conditions may be the result of a combination of the inherent susceptibility of the host (levels of constitutive defence enzymes) and the response of the provenance to external copper, as the rate of copper uptake may vary between provenances. Although seedlings of a *E. globulus* provenance grown under copper adequate conditions may have enhanced production of phenolics and lignification, the extent of this defence response to the pathogen appears to be influenced by the genetics of the plant.

In the current study, tissue copper status did not significantly affect the levels of soluble and bound phenolics. In comparison, Ishaq (1999) reported an increase in phenols and a reduction in lignification in plants grown under copper deficient conditions. Gherardi *et al.* (1999) also observed reduced lignification in *E. globulus* with low tissue copper concentrations. This difference in response to external copper concentrations observed between the current study and those previously, may be the result of the factors used to indicate stress induced by copper deficiency, such as extent of lignification. The method used by Ishaq (1999) and Gherardi *et al.* (1999) to determine extent of lignification involved a phloroglucinol-HCl staining technique, in which changes to stem colour were determined visually. In addition, measurements of lignification may not give a precise indication of a deficiency in copper, as lignification is sensitive to low boron and manganese in eucalypts (Dell *et al.*, 1995). Therefore, more precise measures for assessing stress induced by copper deficiency in *E. globulus* are required.

Gherardi *et al.* (1999) examined the impact of external copper concentrations on the activity of catechol oxidase (a copper-dependant enzyme thought to play a vital role in the biosynthesis of lignin) in *E. globulus* seedlings. The copper treatments were maintained for 20 weeks before young mature leaves (YML) were harvested for measurements of enzyme activity and copper concentration. Symptoms of copper deficiency such as leaf and stem twisting were reportedly evident six to seven weeks after the commencement of the treatments. The results showed that the levels of catechol oxidase were depressed at lower external copper concentrations. In the current study, copper deprived seedlings also displayed typical copper deficient symptoms in the leaves and stems. As the levels of copper in the leaves and stems of *E. globulus* seedlings were comparable in the current study, it may be assumed that symptoms of

copper deficiency would be evident in both tissue types. However, as Gherardi *et al.* (1999) examined catechol oxidase in the YML, it is reasonable to conclude that the patterns of its activity indicate very early changes within the host in response to tissue copper concentration and may not be indicative of the processes occurring within the stem. Examination of catechol oxidase activity in the stems is therefore required. In addition, given that Gherardi *et al.* (1999) indicated a response of *E. globulus* to copper treatments within 20 weeks, it seems unlikely that the duration of the copper treatments in the current trial (1 year) was not sufficient for the deficiency to significantly affect the processes leading to lignification. Further investigation into the role of copper in lignification and identification of the enzymes specific to this role may aid in explaining the discrepancies in the response of *E. globulus* to copper deficiency noted in the current trial and those conducted previously (Gherardi *et al.,* 1999; Ishaq, 1999).

Yu and Rengel (1999) reported that deficiencies in copper, zinc or manganese altered activities of superoxide dismutase (SOD), an enzyme in the early host defence response, indicating that oxidative stress is an outcome of micronutrient deficiency. In addition, a plant subject to deficiency of one nutrient may suffer from toxicity of another nutrient that had been accumulated under stress (Yu and Rengel, 1999). In the current study, there was no statistically significant effect of tissue copper concentration on the activity of PO. A more complete assessment of the micronutrient status of *E. globulus* seedlings (monitoring other key micronutrients such as zinc, manganese or magnesium) may help to explain the patterns of PO activity and lesion development in the current study. In addition, future studies investigating the effect of copper on the cell wall/ plasma membrane interface and reactive oxygen species may provide additional insight into the role of copper in the early plant defence response.

Inoculation was the major factor which influenced the levels of phenolic accumulation and PO activity in stems of *E. globulus* in the current study. A dramatic increase in static and ionic peroxidases was recorded at the lesion front in stems of both *E. globulus* provenances following inoculation with *En. eucalypti*. This increase in PO activity at the lesion front may be an indication of membrane damage (Bowler *et al.*, 1992). In addition, inoculation led to an increase in soluble and bound phenolics throughout the *E. globulus* stems. These results indicate that, regardless of copper status, the *E. globulus* seedlings were capable of inducing a strong defence response to pathogen invasion.

In conclusion, the results of the current study indicate that short term (1 year) external copper conditions in *E. globulus* seedlings do not significantly affect the susceptibility of this host to invasion by *En. eucalypti*. Unlike previous studies (Ishaq, 1999; Gherardi *et al.*, 1999), the current study did not support the hypothesis that copper deficient *E. globulus* have reduced activity of enzymes involved in lignin biosynthesis compared to copper adequate plants. Further studies are required to determine whether the susceptibility of the *E. globulus* seedlings to *En. eucalypti* depends on the ability of the individual provenance to detect the presence of the pathogen and initiate a defence response (including the accumulation of phenolics and an increase in PO activity).

Chapter 8

Does defoliation increase the susceptibility of *Eucalyptus globulus* to *Endothiella eucalypti*?

INTRODUCTION

Defoliation due to insect herbivory or disease has the potential to alter the growth and physiology of host trees through the reduction of the leaf surface area. A reduction in leaf area affects photosynthetic capacity, alters water relations and influences allocation of photosynthates (Chen *et al.*, 2001). The degree to which the host tree is affected by a defoliation event depends on the area of leaf removed, the ability of the tree species to recover from the defoliation event (Chen *et al.*, 2001) and an interaction with other environmental factors, such as drought and temperature (Turner *et al.*, 2001).

Defoliation by disease or insects has been shown to predispose plants to attack by non-aggressive pathogens (Guyon *et al*., 1996; Old *et al.*, 1990; Schoeneweiss, 1981). For example, the susceptibility of four eucalypt species (*Eucalyptus delegatensis, Eucalyptus grandis, Eucalyptus regnans* and *Eucalyptus saligna*) to *Cryphonectria eucalypti* and *Botryosphaeria ribis* was examined before and after defoliation (Old *et al.,* 1990). The study found that, although both fungi were non-aggressive pathogens on vigorously growing seedlings and saplings, they became more damaging after the trees were defoliated. Continuous defoliation further enhanced the development of cankers caused by these fungi (Old *et al.*, 1990). Other studies have shown that the length of time exposed to a defoliation treatment increases the incidence of canker formation by fungi considered to be non-aggressive pathogens, such as *Cytospora* sp. in woody stems (Schoeneweiss, 1981) and *Botryosphaeria ribis* in *Betula alba* (Crist and Schoeneweiss, 1975).

The resistance of the host to withstand defoliation caused by fungi or insects has been correlated with the amount of carbohydrate available for use in the defence response. Carbohydrates are the direct product of photosynthesis, the primary store of energy from which other organic compounds found in plants are synthesised. These carbohydrates may be allocated to current growth and metabolism or to the accumulation of reserves such as starch (Turner *et al.*, 2001; Viiri *et al.*, 2001), which is the principal storage carbohydrate in host hardwoods (Bamber and Humphreys, 1965).

The carbohydrates produced, which are surplus to the above mentioned requirements, are utilised in the synthesis of C-based secondary metabolites such as phenolics, which are key compounds in plant defence (Viiri *et al.*, 2001). The relationship between photosynthesis and carbohydrate metabolism or storage is carefully balanced and easily disturbed (Turner *et al.*, 2001). Therefore, any factor which reduces the size of the canopy in turn affects photosynthetic capacity thereby weakening the resistance of the tree (Viiri *et al.*, 2001).

The increasing incidence of disease caused by canker fungi in *Eucalyptus globulus* plantations in southwestern Australia may be linked to an increase in severity of the leaf disease caused by *Mycosphaerella* spp. (Mycosphaerella leaf disease - MLD) (Maxwell *et al.*, 1998). MLD can significantly reduce the photosynthetic leaf area (especially juvenile leaves) and promote significant loss of leaves. This results in reduced tree growth and increased susceptibility to opportunistic fungi. Previously Paap (2001) investigated the effect of defoliation on the susceptibility of *Corymbia calophylla* to *Endothiella* under glasshouse conditions. This study demonstrated that non-defoliated seedlings had larger lesions when inoculated with *Endothiella* compared to seedlings defoliated to 50 and 100%. This result was unexpected, as it was hypothesised that the greater the percentage of leaves defoliated from plants the more susceptible they would be to canker fungi such as *En. eucalypti*. Preliminary defoliation studies conducted on 18-month-old *E. globulus* seedlings under glasshouse conditions indicated that seedlings defoliated to one-third and two-thirds total leaf number had larger lesions than non-defoliated or completely defoliated trees (Lawrence, 1998). These results raise further questions as to the impact of defoliation on general health and susceptibility of *E. globulus* to *En. eucalypti*. In order to address the processes occurring as a result of defoliation, which may ultimately influence the susceptibility of *E. globulus* to
En. eucalypti, the study reported in this chapter examined the impact of the percentage of defoliation and the duration of defoliation on the susceptibility of *E. globulus* to *En. eucalypti*.

MATERIALS AND METHODS

Effect of four defoliation treatments on the susceptibility of *Eucalyptus globulus* to inoculation with *Endothiella eucalypti*

Experimental design

A randomised complete block design, which consisted of *E. globulus* subjected to five defoliation treatments (0, 50, 75, 90 and 100% of their total leaf number), two inoculation treatments (+/- pathogen) with seven replicate saplings for each treatment combination and a non-defoliated, non-inoculated control, was undertaken in the glasshouse (Steel and Torrie, 1986). Plants were inoculated three weeks after defoliation and harvested 12 weeks after inoculation (Figure 8.1). One stem from each plant was either inoculated with the *En. eucalypti* isolate E81 or a sterile Miracloth® disc.

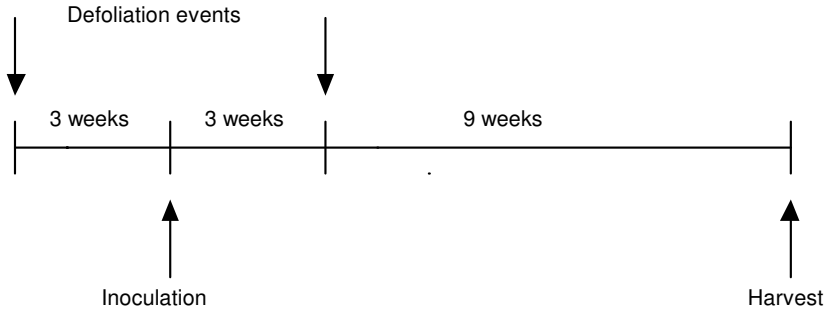


Figure 8.1 Sequence of defoliation and inoculation events undertaken to investigate the impact of defoliation on the susceptibility of *Eucalyptus globulus* to *Endothiella eucalypti*.

Biological materials

Prior to use in the current trial, the *En. eucalypti* isolate E81, was passaged through an excised *E. globulus* stem as described in Chapter 3.

Two-year-old *E. globulus* saplings, supplied by ITC Ltd., were grown in free-draining, 200 mm diameter pots containing potting mix (Chapter 3). The experiment was conducted in a glasshouse (18 - 35 °C min - max) during late spring - early summer (October - January). Plants were watered to field capacity twice a day. Saplings had an average stem diameter of 15 mm at the point of inoculation.

Defoliation treatments

Saplings were defoliated to 50, 75, 90 and 100% on a total leaf number basis. For example, a plant defoliated to 50% had every second leaf removed along the stem and

branches. A plant defoliated to 90% had nine leaves removed for every one remaining. All leaves, including tips, were removed from plants defoliated to 100%. Leaves were removed using fine scissors at the base of the leaf, with care taken not to damage the branch or stem. Leaves removed were collected and leaf area determined using a leaf area measuring machine (Delta T Devices, Hitachi monitor). Seven plants were left non-defoliated. A second defoliation event was undertaken three weeks after inoculation in which all new leaves were removed to maintain the defoliation treatment.

Inoculation and harvest

The plants were inoculated three weeks after the first defoliation event when new shoots were visible. The main stem of each of the *E. globulus* saplings was underbark inoculated with the *En. eucalypti* colonised discs, approximately 20 cm above the base of the stem (as described in Chapter 3). Sterile discs were inserted into the stems as controls.

Twelve weeks after inoculation, the stems were harvested and lesion extension measured. The stems were divided into ten 10 mm segments, the first segment containing the last 10 mm of the lesion (if present) and the nine remaining segments were harvested up the stem (Figure 8.2). If a lesion was not present (in control plants), the stem was divided into ten segments with the first 10 mm containing the point of inoculation. Each of the 10 mm stem segments were cut longitudinally, surface sterilized with 70% ethanol (1-2 sec) and flamed briefly. One half of the 10 mm sections from segments 2, 5 and 10 were stored in 50% ethanol for assessment of starch content and the remaining segments plated onto 1/2PDA+S plates for determination of pathogen colonisation of the stem (Chapter 3). Fungal growth on the plates from each stem segment was monitored over seven days and total colonisation beyond the lesion determined (Chapter 3).

Assessment of starch

The bark was peeled from the stem section stored in 50% ethanol and the inside of the bark thinly hand-sectioned longitudinally, using a sterile single edged blade. Each section was mounted on a microscope slide in iodine. Starch accumulation was assessed according to the number of starch granules visible within ten fields of view (10 x 20 μm^2).

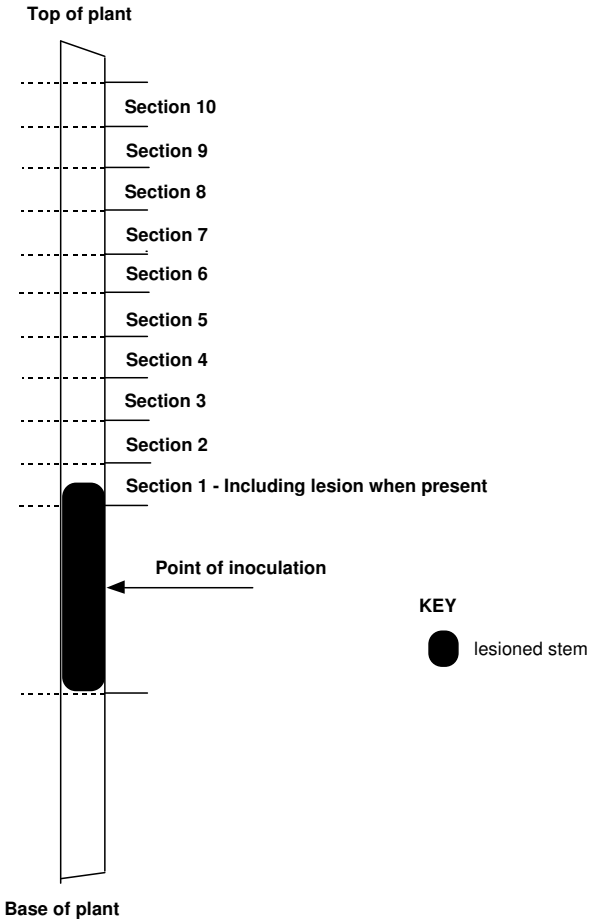


Figure 8.2
Method of harvesting *Eucalyptus globulus* stems after defoliation and inoculation with *Endothiella eucalypti*. Stem sections 1 to 10 were plated onto half strength PDA plates containing streptomycin for determination of pathogen colonisation.

Effect of the duration of defoliation on infection by *Endothiella eucalypti*

Experimental design

The effect of time *E. globulus* plants were exposed to 80% defoliation on their susceptibility to *En. eucalypti* was investigated. Eleven treatments with six replicate plants per treatment were arranged in a randomised complete block design in the glasshouse (Steel and Torrie, 1986). This experiment was designed to build on the results of the previous experiment. Plants were defoliated to 80% total leaf number 0, 2, 4 and 6 weeks prior to inoculation with the *En. eucalypti* isolate E81 or a sterile disc (control) (Figure 8.3). The stem from each plant was inoculated. A set of six non-defoliated, non-wounded plants were also included. Stem diameters and tree height were measured prior to commencement of defoliation treatments, at the time of inoculation (stem diameters only) and at harvest. Measurements of photosynthesis and stomatal conductance were taken before and after inoculation. Carbohydrate levels in the stem were measured at harvest.

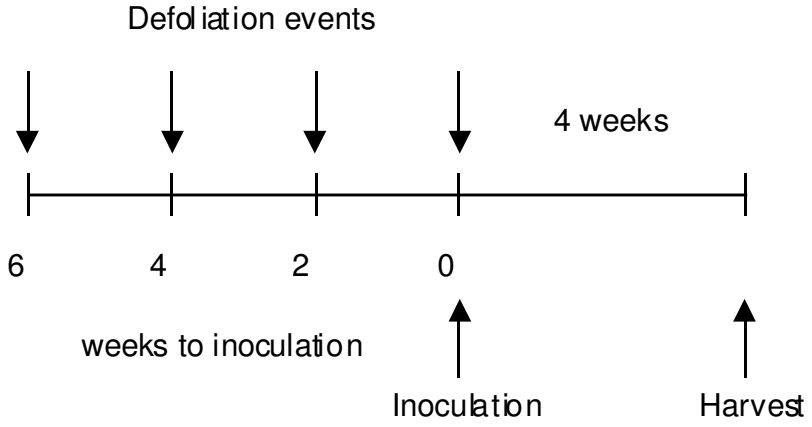


Figure 8.3 Sequential defoliation of *Eucalyptus globulus* (weeks), time of inoculation with *Endothiella eucalypti* and final harvest.

Biological materials

The *En. eucalypti* isolate E81 was passaged though an excised *E. globulus* stem prior to use in the current study (Chapter 3).

One-year-old *E. globulus*, supplied by ITC Ltd, were grown under conditions outlined above, in an evaporatively-cooled glasshouse (20-27 °C min-max). Plants were watered to field capacity via a dripper irrigation system for 10 minutes four times a day.

Defoliation treatments

Plants were defoliated to 80% on a leaf number basis in accordance with findings of the previous defoliation trial. The leaves were removed as outlined above, collected and weighed. Plants were maintained at 80% defoliation by removing any new leaf growth every 7-10 days. A set of 18 plants were left non-defoliated.

Inoculation and harvest

The main stem was underbark inoculated with the *En. eucalypti* colonised discs as described in the previous defoliation trial. Stems were inoculated approximately 750 mm above the base of the plant. Sterile discs were inserted into the stem as controls.

Twenty-eight days after inoculation, plants were harvested and stem diameters and lesions measured. Stems were cut into 10 mm pieces and plated onto 1/2PDA+S plates

as described earlier. A 30 mm section of the stem was collected for carbohydrate analysis from 100 mm above the lesion front (Figure 8.4). The bark was removed from the wood, material frozen individually in liquid nitrogen and stored at -80 °C for carbohydrate analysis. Remaining leaves were removed and weighed.

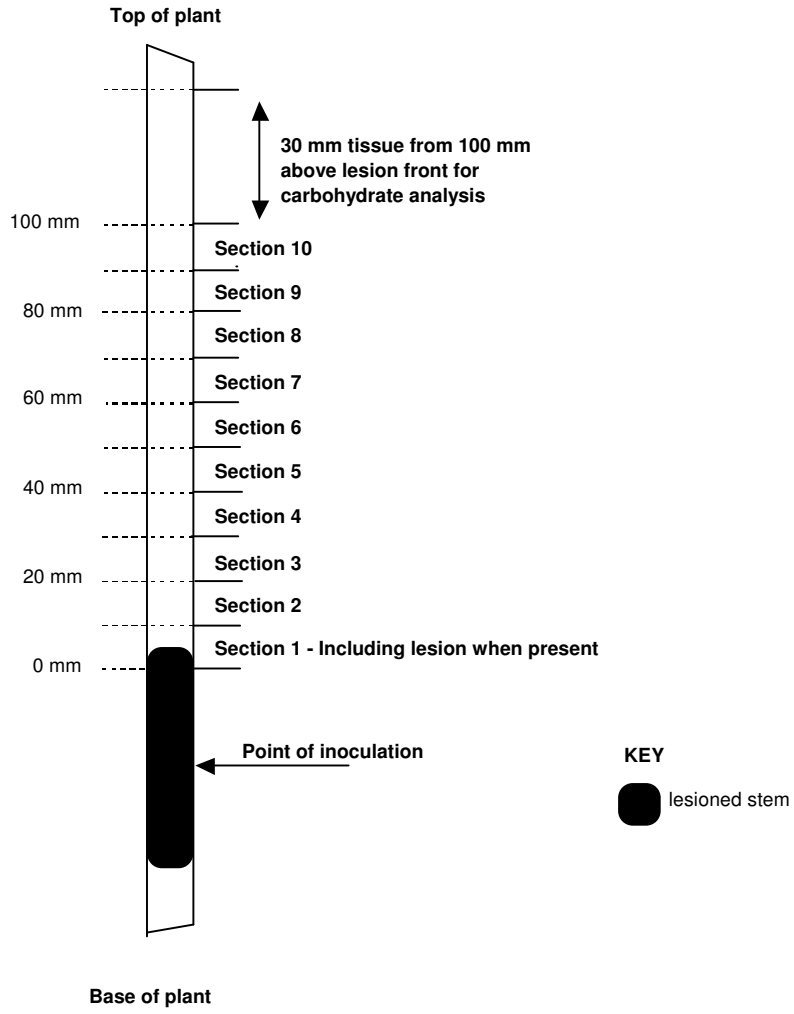


Figure 8.4 Method of harvesting *Eucalyptus globulus* stems after defoliation and/or inoculation with *Endothiella eucalypti*. Stem sections 1 to 10 were plated onto half strength PDA plates containing streptomycin for determination of pathogen colonisation. The 30 mm section 100 mm above lesion front was used for determination of stem carbohydrate content.

Photosynthesis

Measurements of photosynthesis (CO_2 assimilation) and stomatal conductance were taken under conditions of full sun using a CIRAS-2 machine (PPSystems, www.ppsystems.com) (Figure 8.5). Settings included cuvette environment: supplied CO_2 concentration 370 ppm, H_2O = 100% Ambient, PAR=1000. Initially measurements were taken between the hours of 9.30 and 15.30 to determine the period during which the *E. globulus* were most photosynthetically active (Figure 8.6). Photosynthesis and stomatal conductance were then recorded between the hours of 11.30 and 14.30.

Figure 8.5
CIRAS-2 machine used to determine assimilation and stomatal conductance in *Eucalyptus globulus*.

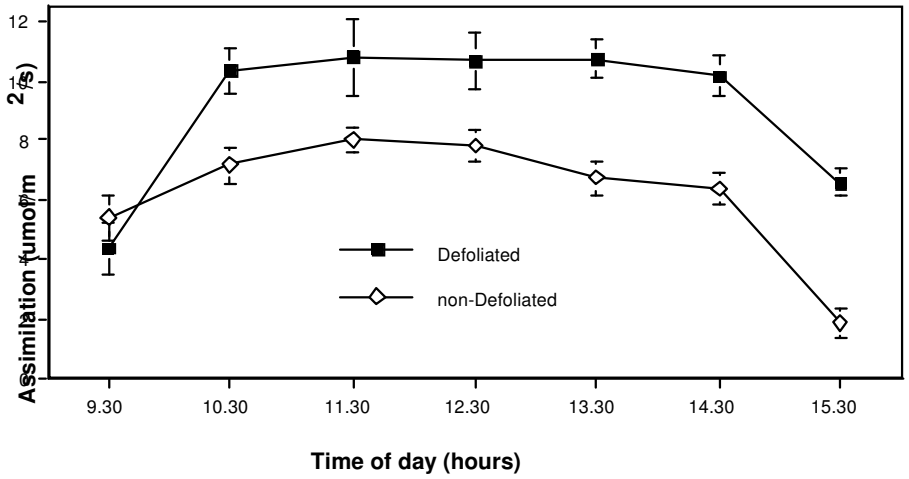


Figure 8.6 Rate of CO_2 assimilation (µmol/m^2/s) for leaves of *Eucalyptus globulus* defoliated to 80% of total leaf number compared with non-defoliated trees between the hours of 9.30 and 15.30.

Non-structural carbohydrate analysis

Soluble and insoluble carbohydrate fractions were determined using the methods outlined by Hansen and Moller (1975) and Yemm and Willis (1954). Soluble and insoluble carbohydrate fractions were extracted from 20 - 50 mg of finely ground bark tissue. Bark was ground (Breville Coffee grinder; Breville Holdings Pty. Ltd. NSW) and then powdered in liquid nitrogen, weighed into 10 mL polyethylene tubes and stored at -20 °C. To each of the bark samples, 5 mL of distilled water was added and the mixture boiled at 100 °C for 30 min in a waterbath. The tubes were then centrifuged at 4 000 *g* for 20 min and the supernatant decanted into a new 10 mL tube. Another 5 mL of distilled water was added to the pellet and the boiling, centrifuging and decanting processes repeated. The 10 mL supernatant containing the soluble carbohydrate fraction was then stored at -20 °C until analysis. To the remaining pellet, 5 mL of 1.1% HCl was added and the mixture boiled at 100 °C for 30 min. After boiling, an additional 5 mL of 1.1% HCl was added to the tube containing pellet and supernatant. The mixture was again boiled at 100 °C and centrifuged as described previously. The tube containing the pellet and supernatant containing the insoluble carbohydrate portion was then stored at -20 °C until analysis.

Anthrone reagent (1g Anthrone; Sigma A-1631, in 500 mL 72% sulfuric acid) was prepared and stored on ice. A 1 mL aliquot of each of the soluble and insoluble carbohydrate portions was pipetted into a glass reaction tube and placed on ice. The

anthrone reagent (5 mL) was added to each tube, the solution, vortexed and stored on ice. Samples were then incubated at 100 °C for 11 minutes to commence the reaction with the anthrone reagent. After this time, the tubes were placed on ice to terminate the reaction. The absorbance of the resultant solutions was then determined at 630 nm within one hour of terminating the reaction. The soluble and insoluble carbohydrate concentrations were determined according to a standard curve prepared using starch standards (Sigma T-4762) diluted in 1.1% HCl.

Leaf area

Due to failure of the machine used in the previous trial to assess leaf area, leaf area for the current trial was assessed as follows. From pre-weighed leaf samples taken from each plant, a subsample of 10% of the total leaf weight was removed from each tree for calculation of leaf area. Leaves were attached to A4 paper (120 x 295 mm), pressed, then scanned (HP Scanjet 9200) into a computer. Leaf area was analysed using Jasc® Paint Shop Pro™ (Jasc Software, USA Version 7.02).

Statistical analysis

Data obtained for lesion extension, colonisation, stem diameter, height, leaf area, carbohydrate, starch and assimilation/stomatal conductance were analysed separately by ANOVA using Statistica Version 4.1 (StatSoft® Inc., OK, USA). Data were assessed for homogeneity, variation of the mean from the variance and fit to a normal distribution. In the first defoliation trial, means for lesion extension and total colonisation were compared by LSD ($p \leq 0.01$), and stem diameter and starch at $p \leq 0.05$. Leaf area was log transformed to meet statistical requirements and means compared by LSD ($p \leq 0.01$). Leaf area, lesion extension and colonisation data recorded in the second defoliation trial were log transformed to meet statistical requirements with means compared by LSD ($p \leq 0.05$). Data for stem diameter, tree height and stomatal conductance were compared by LSD ($p \leq 0.05$) and photosynthetic assimilation and carbohydrate content was compared by LSD ($p \leq 0.01$). All data was presented with standard error of the mean.

RESULTS

Effect of four defoliation treatments on the susceptibility of *Eucalyptus globulus* to inoculation with *Endothiella eucalypti*

Effect of defoliation on plant growth

The defoliation treatments resulted in evenly distributed leaf removal, with a visual difference in remaining leaf area after the four defoliation treatments. There was a significant ($p<0.0001$) difference in the amount of leaf area removed from the *E. globulus* saplings during the four defoliation treatments (Table 8.1; Figure 8.7A). The new leaf growth stimulated as a result of defoliation was predominantly distributed at the tips of the branches. New leaves, in some cases, showed signs of chlorosis and often had a curved leaf edge. Trees defoliated to 100% were dominated by apical clusters of small leaves which were predominantly healthy (Figure 8.8). Two trees defoliated to 100% coppiced from the base, one of which was stunted in its growth. Tip dieback was recorded on one of the 100% defoliated trees on branches interconnecting with the infected stem. At the time of harvest the older leaves of trees defoliated to 75% and above were yellow/red in colour and few remained at the time of harvest.

Table 8.1 ANOVA of Log leaf area removed (cm^2) from *Eucalyptus globulus.* Significant value in bold font.

Effect	MS Effect	MS Error	F (df 4, 30)	P
Defoliation	99.295	0.164	605.499	**<0.0001**

Stem diameter was significantly ($p=0.003$) smaller in defoliated trees compared to non-defoliated trees (Table 8.2). The stem diameters of non-defoliated (0%) trees were significantly ($p=0.019$, $p=0.01$, $p=0.001$ and $p=0.0002$) greater than trees at 50, 75, 90 and 100% defoliation, respectively (Figure 8.7B).

Table 8.2 ANOVA of stem diameter (mm) of *Eucalyptus globulus* defoliated at 0, 50, 75, 90 and 100% total leaf number and inoculated with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df 4, 30)	P
Defoliation	32.352	6.205	5.214	**0.003**

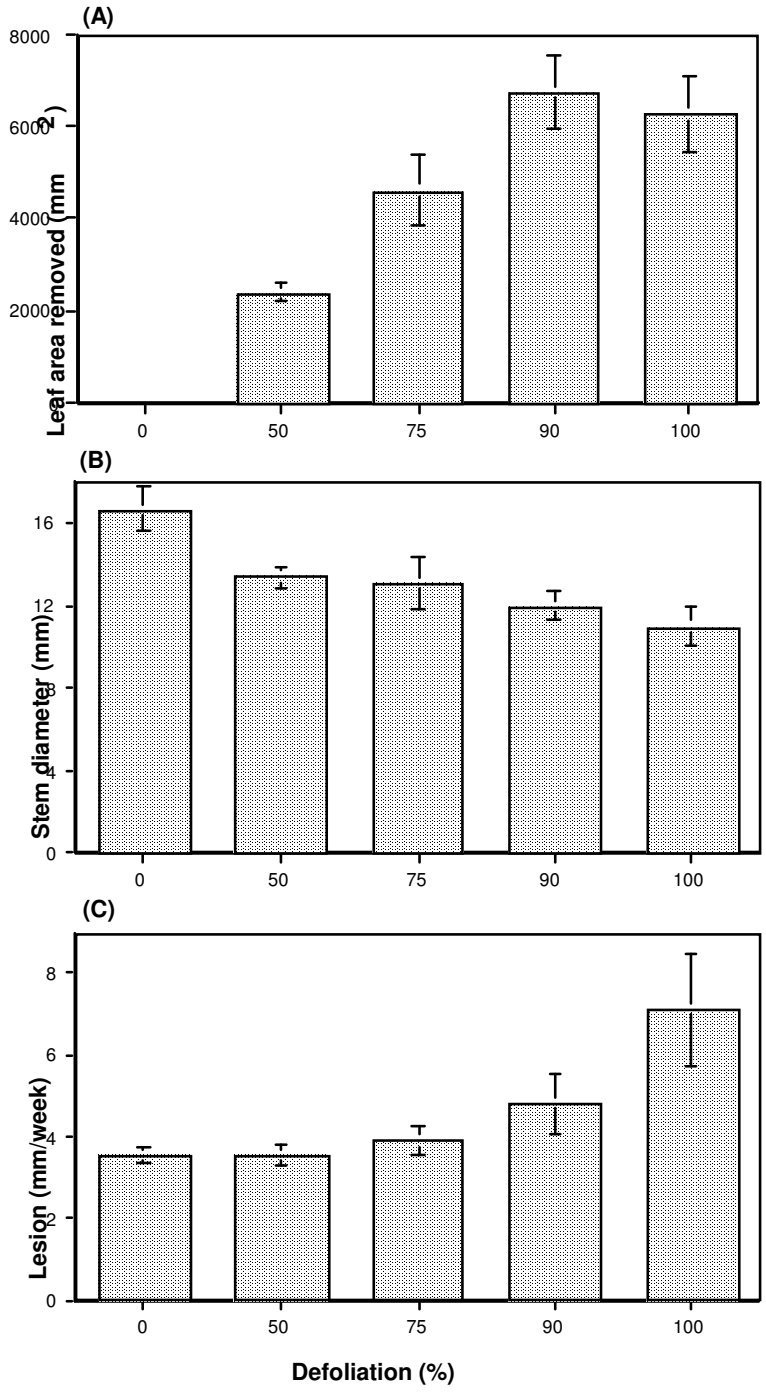


Figure 8.7 (A) Leaf area (cm^2) removed from *Eucalyptus globulus* during defoliation, (B) stem diameter (mm) and (C) stem lesion extension (mm/ week) in saplings defoliated to 0, 50, 75, 90 and 100% total leaf number and inoculated with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

Figure 8.8
Regrowth on two-year-old *Eucalyptus globulus* three weeks after complete (100%) defoliation. Note that new growth is small and predominately distributed in clusters at the ends of the branches.

Disease development

Lesion extension (mm/week) and total colonisation (mm/week) increased significantly (p=0.009 and p=0.01, respectively) in defoliated trees (Table 8.3). Lesion extension and total colonisation were highly correlated (r=1.0) (data not shown), therefore lesion data only are discussed in detail. Stem lesions in trees defoliated to 100% were significantly (p=0.003, p=0.002 and p=0.004) longer than those from 0, 50 and 75% defoliated trees, respectively (Figure 8.7C). Greater than 75% defoliation was required to effect lesion extension (Figure 8.7C). There was no correlation between lesion extension (mm/week) and stem diameter (r=0.216) (data not shown). In addition, there was no correlation between leaf area removed from trees and lesion extension (mm/week) (r=0.047) or stem diameter (r=0.292) (data not shown).

Table 8.3 ANOVA of (A) lesion extension (mm/week) and (B) total colonisation (mm/week) in stems of *Eucalyptus globulus* defoliated at 0, 50, 75, 90 and 100% total leaf number and inoculated with *Endothiella eucalypti*. Significant values in bold font.

Effect	MS Effect	MS Error	F (df 4, 28)	P
Defoliation (A)	15.785	3.809	4.144	**0.009**
Defoliation (B)	15.486	3.830	4.044	**0.010**

Endothiella eucalypti was not recovered beyond the lesion front (stem section 2 shown in Figure 8.2) in any of the defoliation treatments. However, it was recovered most frequently from the first segment in trees defoliated to 50% (71% of cases). For the remaining defoliation treatments (0, 50, 90 and 100%) recovery of *En. eucalypti* from segment 1 was generally low and occurred in 33, 43, 28 and 14% of cases, respectively.

Non-defoliated trees or those with 50% of leaves removed were more likely to exhibit swelling of the stem following inoculation (34% and 50% of trees, respectively) than those more heavily defoliated (14%, 28% and 0% in trees defoliated to 75, 90 and 100%, respectively) (Table 8.4; Figure 8.9). Completely defoliated trees had the largest proportion of pycnidia present on the stem at the time of harvest (Table 8.4).

Table 8.4 Percentage of stems of *Eucalyptus globulus* defoliated at 0, 50, 75, 90 and 100% total leaf number exhibiting symptoms of kino exudation, stem swelling, pycnidia formation, stem girdling and cracking in response to inoculation with *Endothiella eucalypti*.

Defoliation (%)	Kino present (%)	Swelling of stem (%)	Trees with pycnidia present (%)	Girdling of stem (%)	Cracking of stem (%)
0	67	34	34	50	84
50	67	50	34	84	100
75	100	14	0	57	100
90	14	28	28	57	43
100	14	0	86	50	86

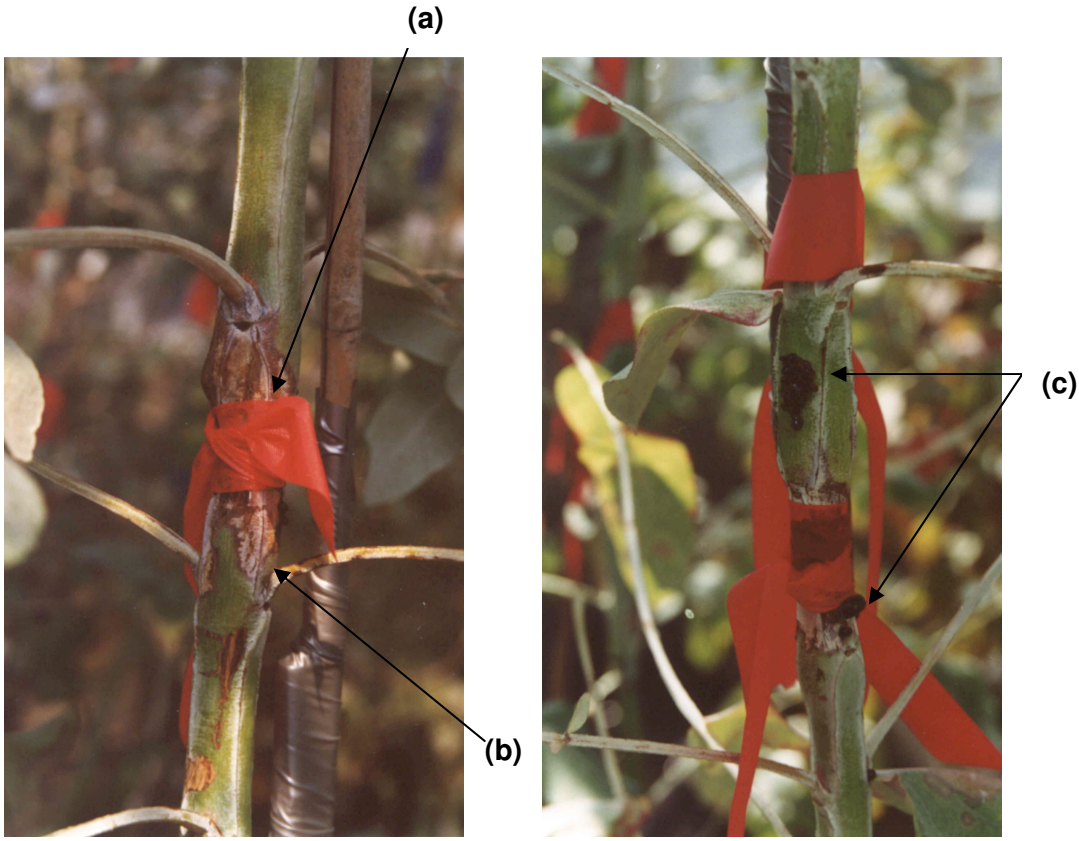


Figure 8.9 Lesion development in stems of one-year-old *Eucalyptus globulus* wound inoculated with *Endothiella eucalypti* showing (a) swelling of the stem, (b) stem whitening and (c) kino exudation.

Starch accumulation

Starch accumulated in the living rays of the bark tissue. Starch grains were unevenly distributed throughout the bark tissue, often aggregated into clusters. Defoliation did not significantly (p=0.095) affect on the accumulation of starch grains in the inner bark of inoculated *E. globulus* stems (non-defoliated 62.46 ± 7.08 grains/ 20 µm^2 compared to 100.87 ± 19.68 grains/ 20 µm^2 defoliated; Table 8.5A). In contrast, inoculation significantly (p=0.001) decreased the number of starch granules in non-defoliated trees (non-inoculated 135.20 ± 18.10 grains/ 20 µm^2 compared to inoculated 62.46 ± 7.08 grains/ 20 µm^2; Table 8.5B).

Table 8.5 ANOVA of starch grains (number of grains/20 µm^2) in bark of (A) non-defoliated and 100% defoliated *Eucalyptus globulus* inoculated with *Endothiella eucalypti* and (B) non-defoliated *E. globulus* before and after inoculation with *En. eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df 1, 26)	P
Defoliation (A)	10272.00	3429.575	2.995	0.095
Inoculation (B)	36847.23	2946.909	12.504	**0.001**

Effect of the duration of defoliation on infection by *Endothiella eucalypti*

Over the trial period, the older leaves turned yellow and red in colour and were naturally defoliated from the trees (Figure 8.10). This natural defoliation did not affect on the overall leaf area of the trees and was relatively uniform for all treatments.

Figure 8.10 Older leaves from defoliated trees developed a yellow and red colouring and were eventually naturally defoliated from the trees.

The leaf area remaining on the trees for the duration of the inoculation period following defoliation treatments was significantly ($p<0.0001$) larger in the non-defoliated trees compared to those defoliated to 80% (Figures 8.11 and 8.12; Table 8.6).

Table 8.6 ANOVA of leaf area (cm^2) remaining at harvest on *Eucalyptus globulus,* non-defoliated and defoliated to 80% total leaf number for the duration of time inoculated with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df 4, 55)	P
Defoliation	5.864	0.030	197.66	**<0.0001**

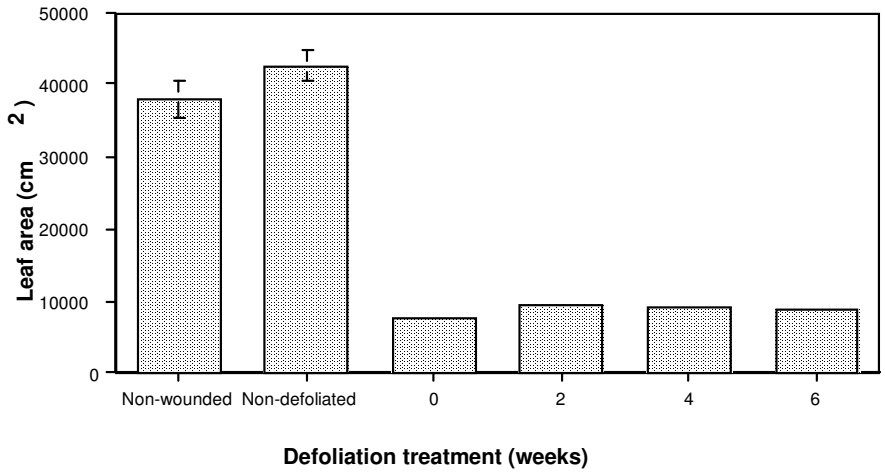


Figure 8.11 Leaf area (cm^2) remaining at harvest on *Eucalyptus globulus,* non-defoliated and defoliated to 80% 6, 4, 2, and 0 weeks prior to inoculation with *Endothiella eucalypti.* Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal.

Figure 8.12 One-year-old *Eucalyptus globulus* trees prior to (A) and after (B) defoliation to 80% of total leaf number.

Effect of defoliation and inoculation on plant growth

Prior to the defoliation treatments, the heights of the plants were relatively uniform (Figure 8.13). Defoliation did not have a significant effect on the vertical growth of *E. globulus* (p=0.173) (Table 8.7). However at harvest (ten weeks after the establishment of defoliation treatments), inoculated seedlings were significantly (p=0.045) smaller than non-inoculated plants (Table 8.7; Figure 8.13).

Table 8.7 ANOVA of height (cm) of *Eucalyptus globulus* non-defoliated and defoliated 6, 4, 2, and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	138336.5	83122.00	1.664 (4, 50)	0.173
Inoculation (2)	349606.7	83122.00	4.206 (1, 50)	**0.045**
1 x 2	15905.6	83122.00	0.191 (4, 50)	0.942

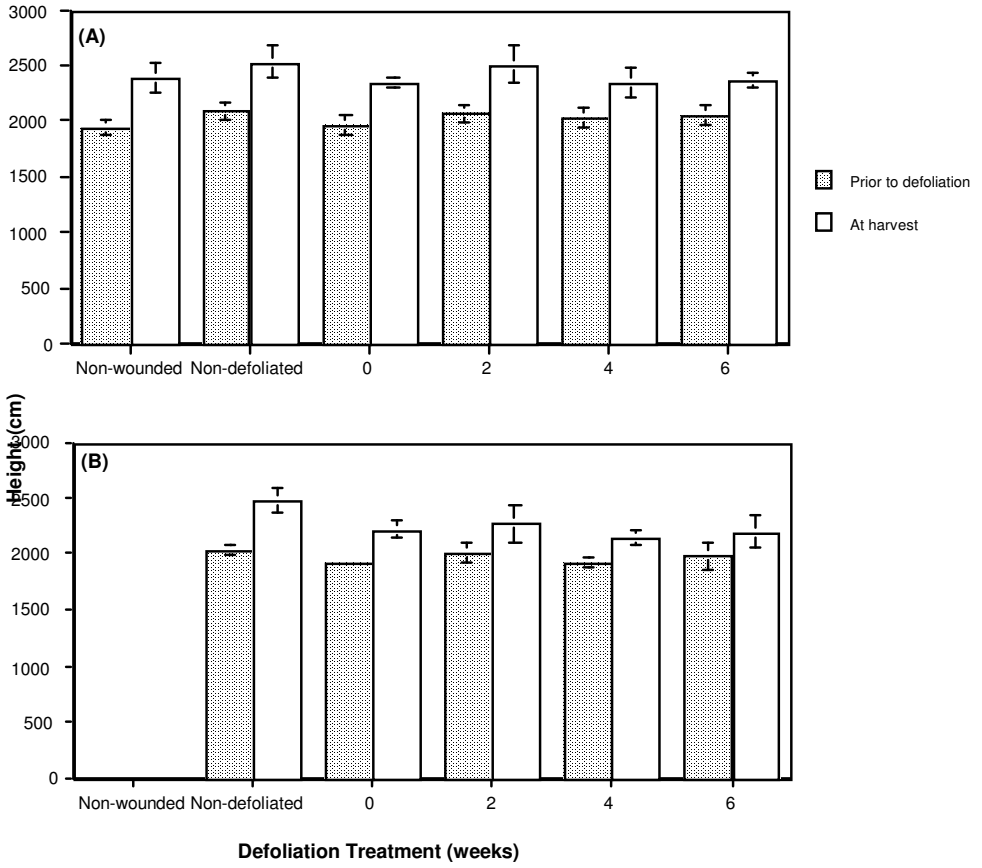


Figure 8.13 Height (cm) of (A) non-inoculated and (B) inoculated *Eucalyptus globulus* trees prior to defoliation treatments and inoculation with *Endothiella eucalypti* and at harvest. Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal. Missing data in (B) indicates non-wounded plants which were not inoculated as this would void treatment.

As observed for tree height, stem diameter was relatively uniform prior to commencement of the defoliation treatments (Table 8.8; Figure 8.14). At the time of inoculation (six weeks after the initiation of defoliation treatments), defoliated trees had significantly ($p<0.0001$) smaller stem diameters compared to non-defoliated trees (Table 8.9). At this time, non-defoliated plants had significantly ($p=0.02$, $p=0.04$, $p<0.0001$ and $p<0.0001$) larger stem diameters compared to plants defoliated to 80% total leaf number 0, 2, 4, and 6 weeks prior to inoculation, respectively (Figure 8.14). The stem diameter of plants which had been defoliated for six weeks were also significantly ($p<0.0001$ and $p=0.0007$) smaller compared to those defoliated two weeks and defoliated at the time of inoculation, respectively.

Table 8.8 ANOVA of stem diameter (mm) of *Eucalyptus globulus* prior to defoliation.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	1.548	1.479	1.046 (4, 50)	0.393
Inoculation (2)	0.337	1.479	0.228 (1, 50)	0.635
1 x 2	0.745	1.479	0.504 (4, 50)	0.733

Table 8.9 ANOVA of stem diameter (mm) of *Eucalyptus globulus* non-defoliated and defoliated 0, 2, 4, and 6 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	17.971	1.642	10.944 (4, 49)	**0.000**
Inoculation (2)	2.184	1.642	1.330 (1, 49)	0.254
1 x 2	2.201	1.642	1.340 (4, 49)	0.268

At the time of harvest, there was a significant ($p=0.035$) interaction between the timing of defoliation events and inoculation on stem diameters of *E. globulus* (Table 8.10). Defoliated plants had significantly ($p<0.0001$) smaller stem diameters compared to non-defoliated trees (Table 8.10) with the stem diameter reducing with increasing time of defoliation (Figure 8.14).

Table 8.10 ANOVA of stem diameter (mm) at the time of harvest of *Eucalyptus globulus* non-defoliated and defoliated 0, 2, 4, and 6 weeks prior to inoculation with *Endothiella eucalypti* and harvested after four weeks. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	30.914	2.121	14.578 (4, 50)	**0.000**
Inoculation (2)	0.641	2.121	0.302 (1, 50)	0.585
1 x 2	5.981	2.121	2.820 (4, 50)	**0.035**

At the time of harvest, inoculated stems were larger than non-inoculated stems in defoliated trees but smaller in non-defoliated plants (Figure 8.14). Stem diameter appeared visually smaller at the point of inoculation compared to outside the lesioned area, however the diameter at the point of inoculation was equal to, or greater than, the diameter recorded prior to inoculation. At harvest, the stem diameters of wounded stems were greater compared to non-wounded stems (Figure 8.14).

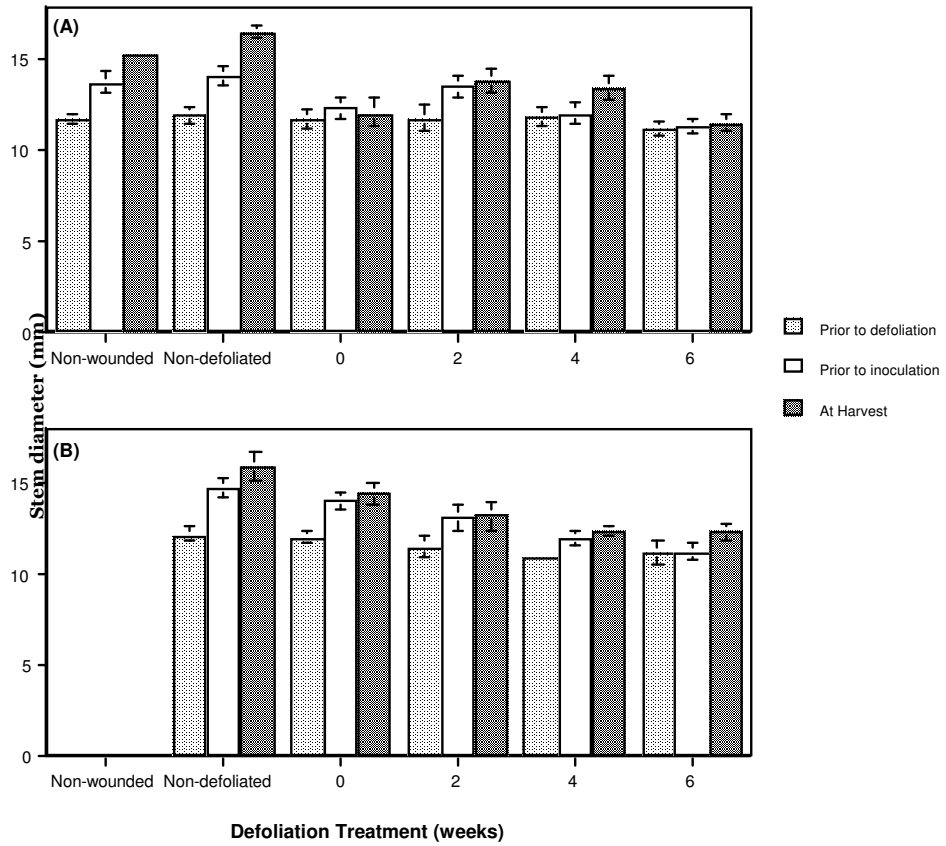


Figure 8.14 Stem diameters (mm) of (A) control non-inoculated or (B) inoculated *Eucalyptus globulus* prior to commencement of defoliation treatments, prior to inoculation with *Endothiella eucalypti* and four weeks later at harvest. Trees had either undergone defoliation to 80% of total leaf number 0, 2, 4, and 6 weeks prior to inoculation or remained non-defoliated and non-wounded. Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal. Missing data in (B) indicates non-wounded plants which were not inoculated as this would void treatment.

Disease development

Stem cracking, exudation of kino, stem whitening (white colouration of the stem) and the presence of pycnidia were all found, to varying degrees, in association with lesions on *E. globulus* stems inoculated with *En. eucalypti*, regardless of the defoliation treatment (Table 8.11). Pycnidia were most commonly recorded on stems of trees defoliated four weeks prior to inoculation (83%) and kino exudation was associated with 100% of the stems defoliated two weeks prior to inoculation (Table 8.11).

Table 8.11 Observations of changes to stems of non-defoliated *Eucalyptus globulus* trees and those defoliated 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Observations were recorded four weeks after inoculation.

Time of defoliation to 80% (weeks)	% of plants with pycnidia present	% of plants with stem whitening near lesion	% of plants with stem cracking in lesion	% of plants with kino exudation from lesion
Non defoliated	66	66	100	83
0	50	66	83	83
2	66	100	50	100
4	83	16	50	100
6	66	66	33	83

Overall, defoliation treatments did not significantly (p=0.067 and p=0.056) increase lesion extension or total stem colonisation, respectively, of *E. globulus* inoculated with *En. eucalypti* (Table 8.12). As there was a high correlation (r=0.944) between lesion extension (mm/week) and total colonisation of the *E. globulus* stems (Figure 8.15), lesion extension only will be discussed in detail.

Table 8.12 ANOVA of (A) Log lesion extension (mm/week) and (B) Log colonisation (mm/week) in stems of *Eucalyptus globulus* non-defoliated and defoliated 0, 2, 4 and 6 weeks prior to inoculation with *Endothiella eucalypti*.

Effect	MS Effect	MS Error	F (df 4, 21)	P
Defoliation (A)	0.086	0.033	2.579	0.067
Defoliation (B)	0.078	0.029	2.739	0.056

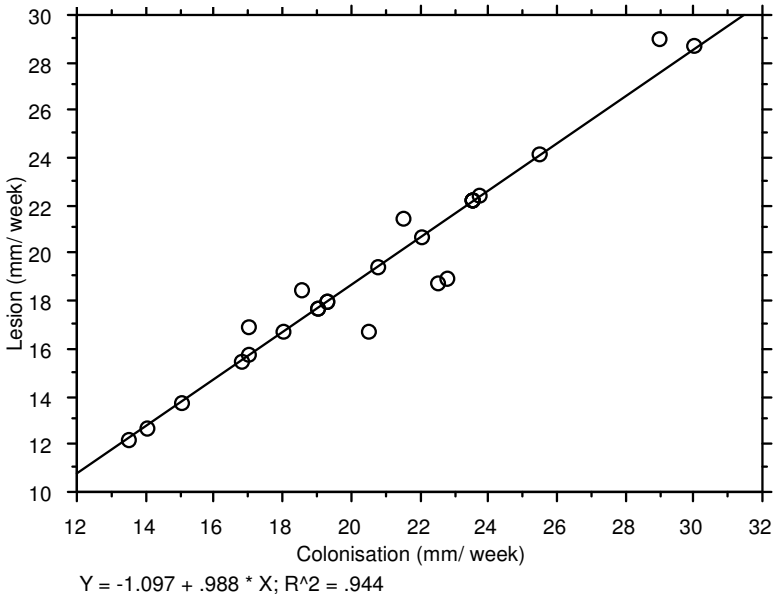


Figure 8.15 Correlation between lesion extension (mm/ week) and stem colonisation (mm/ week) in stems of *Eucalyptus globulus* either non-defoliated or defoliated to 80% of total leaf number 6, 4, 2, and 0 weeks prior to inoculation with *Endothiella eucalypti*.

Although defoliation did not have a significant impact on lesion extension overall, plants defoliated six weeks prior to inoculation did have significantly (p=0.006, p=0.04 and p=0.047) larger lesions compared to non-defoliated plants, those defoliated immediately prior to and four weeks prior to inoculation, respectively (Figure 8.16).

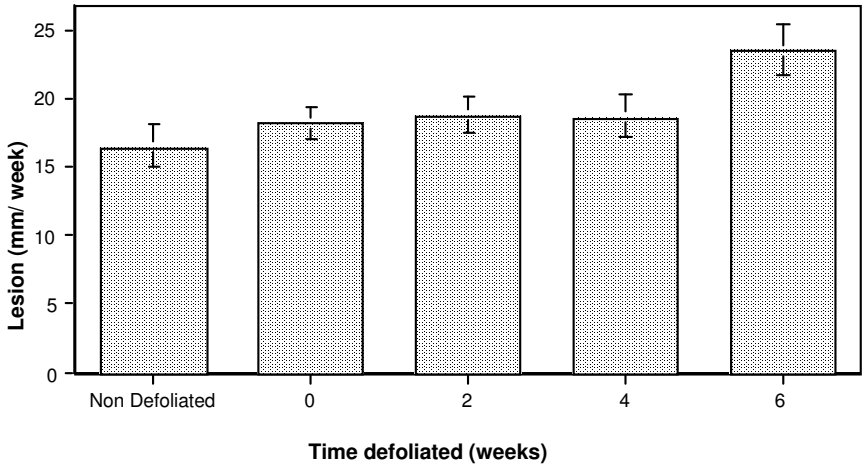


Figure 8.16 Lesion extension (mm/week) in stems of *Eucalyptus globulus* either non-defoliated or defoliated to 80% of total leaf number 6, 4, 2, and 0 weeks prior to inoculation with *Endothiella eucalypti*. Bars indicate standard errors of the mean.

Although there was no significant difference ($p \leq 0.05$) overall between defoliated and non-defoliated trees, the length of time the trees were defoliated did impact on the extent of lesion development. Therefore an analysis was conducted using defoliated tress only. Within defoliation treatments, the timing of defoliation did not significantly (p=0.121) affect lesion extension (Table 8.13), however plants defoliated six weeks prior to inoculation did have significantly (p=0.040) larger lesions compared to plants defoliated at the time of and four weeks prior to inoculation (Figure 8.16). There was no correlation (r=0.125) between lesion extension and leaf area remaining on the tree during the period of inoculation (data not shown).

Table 8.13 ANOVA of log lesion extension (mm/week) in stems of *Eucalyptus globulus* defoliated 0, 2, 4 and 6 weeks prior to inoculation with *Endothiella eucalypti*.

Effect	MS Effect	MS Error	F (df 3, 17)	P
Defoliation timing	0.071	0.032	2.232	0.121

Photosynthesis

The maximum rate of CO_2 assimilation in defoliated *E. globulus* occurred between the hours of 11.30 and 14.30 and from 11.30 and 12.30 in non-defoliated trees (Figure 8.6). The defoliated trees had a significantly (p=0.0001) higher rate of photosynthesis than the non-defoliated trees (Table 8.14). All later photosynthesis measurements were recorded between the hours of 11.30 and 14.30.

Table 8.14 ANOVA of CO_2 assimilation (µmol/m^2/s) of leaves of non-wounded/ non-inoculated *Eucalyptus globulus* trees and those which had been defoliated to 80% total leaf number for six weeks. Readings were taken hourly over a period of one day between the hours of 9.30 and 15.30. Significant value in bold font.

Effect	Rao's R (df 7, 10)	P
Defoliation	15.001	**0.0001**

Defoliation significantly (p<0.0001) increased assimilation rate and stomatal conductance in both inoculated and non-inoculated *E. globulus* (Tables 8.15 to 8.18; Figure 8.17). Inoculation did not have a significant (p=0.093 and p=0.228) affect on photosynthesis or stomatal conductance, respectively (Tables 8.17 and 8.18). However, there was a significant (p=0.005) interaction for stomatal conductance between defoliation and inoculation (Table 8.16; Figure 8.18). No significant (p=0.418 and p=0.256) difference in assimilation rates or stomatal conductance, respectively was recorded between the trees defoliated just prior and six weeks prior to inoculation (Figure 8.17). Wounding did not significantly (p=0.943 and p=0.394) affect assimilation rate or stomatal conductance, respectively in non-defoliated trees (Table 8.19; Figure 8.17).

Table 8.15 ANOVA of CO_2 assimilation (µmol/m^2/s) of leaves of non-inoculated *Eucalyptus globulus* non-defoliated/wounded, non-defoliated/non-wounded and defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior. Significant value in bold font.

Effect	MS Effect	MS Error	F (df 3, 41)	P
Defoliation	149.69	6.952	21.531	**<0.0001**

Table 8.16 ANOVA of stomatal conductance (mmol/m^2/s) of leaves of non-inoculated *Eucalyptus globulus* non-defoliated/wounded, non-defoliated/non-wounded and defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior. Significant value in bold font.

Effect	MS Effect	MS Error	F (df 3, 40)	P
Defoliation	304699.1	27227.2	11.19	**<0.0001**

Table 8.17 ANOVA of CO_2 assimilation (µmol/m^2/s) *Eucalyptus globulus* defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	343.329	6.232	55.090 (2, 66)	**<0.0001**
Inoculation (2)	18.100	6.232	2.904 (1, 66)	0.093
1 x 2	7.159	6.232	1.149 (2, 66)	0.323

Table 8.18 ANOVA of stomatal conductance (mmol/m^2/s) *Eucalyptus globulus* defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant values in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	974926.3	23157.02	42.10 (2, 66)	**<0.0001**
Inoculation (2)	34278.3	23157.02	1.480 (1, 66)	0.228
1 x 2	134927.4	23157.02	5.827 (2, 66)	**0.0047**

Table 8.19 ANOVA of (A) CO_2 assimilation (µmol/m^2/s) and (B) stomatal conductance of leaves of non-wounded and wounded non-defoliated *Eucalyptus globulus*.

Effect	MS Effect	MS Error	F (df)	P
Wounding (A)	0.043	8.221	0.005 (1, 19)	0.943
Wounding (B)	17184.13	22583.31	0.761 (1, 18)	0.394

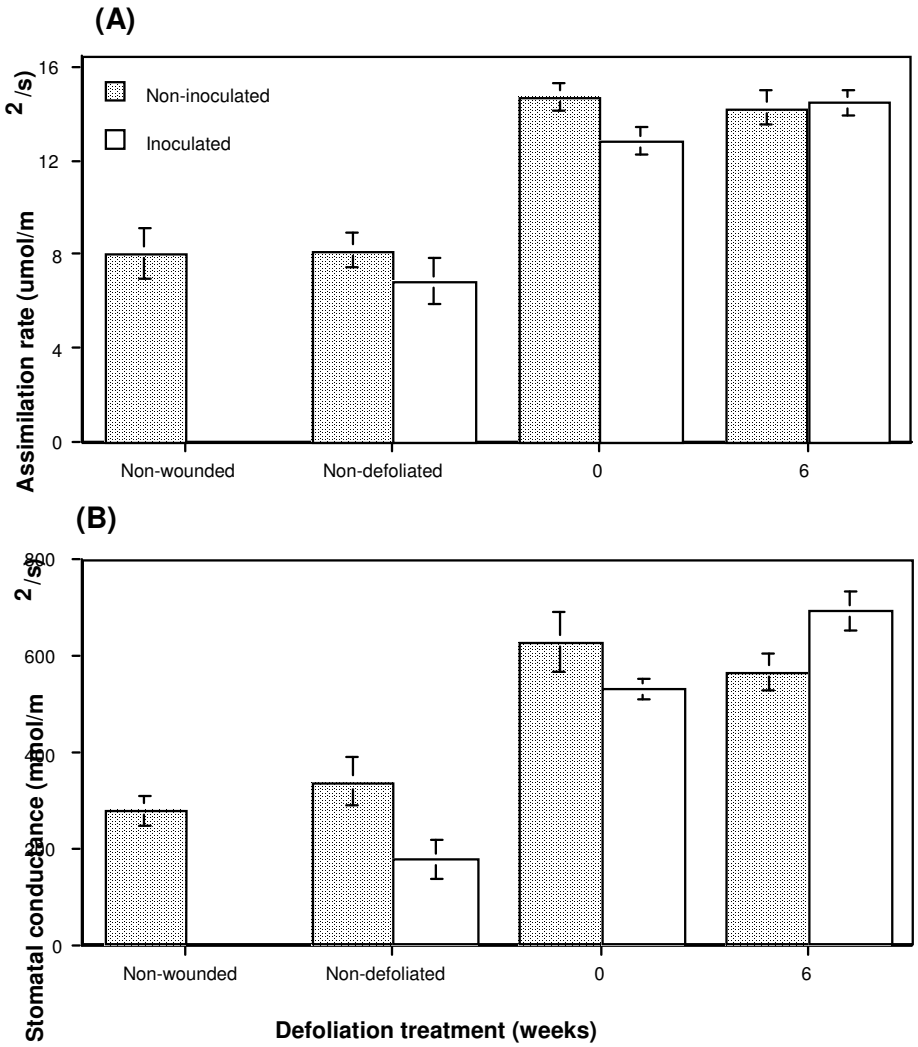


Figure 8.17 (A) CO_2 assimilation rate (µmol/m^2/s) and (B) stomatal conductance (mmol/m^2/s) of leaves of non-defoliated *Eucalyptus globulus* and those trees defoliated at the time of inoculation and six weeks prior to inoculation with *Endothiella eucalypti*. Measurements were also taken from leaves of trees non-wounded, inoculated and non-inoculated wounded controls. Bars indicate standard errors of the mean. Missing data in (B) indicates non-wounded plants which were not inoculated as this would void treatment.

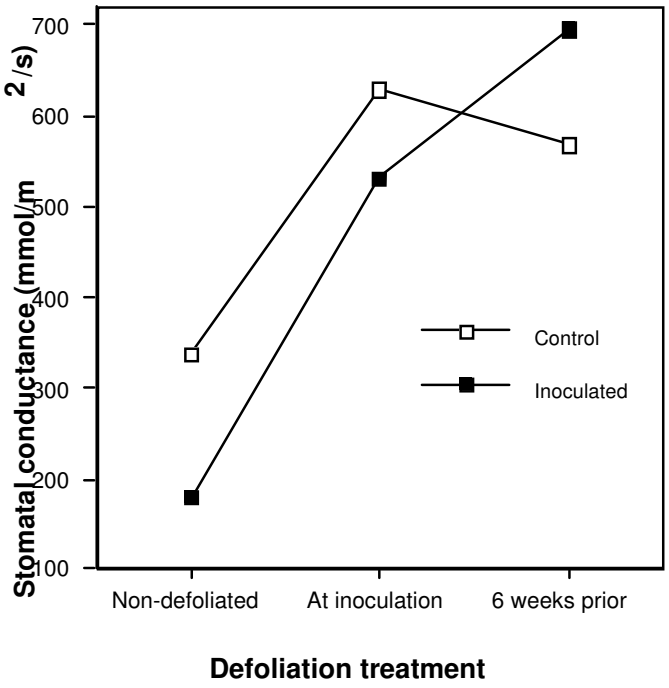


Figure 8.18 Two-way interaction plot (f (2, 66)=5.83: p<0.0047) for stomatal conductance (mmol/m^2/s) of *Eucalyptus globulus* leaves of non-defoliated *Eucalyptus globulus* and those trees defoliated at the time of inoculation and six weeks prior to inoculation with *Endothiella eucalypti*.

Carbohydrate analysis

Defoliation treatment had a significant (p<0.0001) impact on the levels of soluble carbohydrates within the bark of *E. globulus* (Table 8.20; Figure 8.19). The concentrations of insoluble carbohydrates in the bark of inoculated plants was significantly (p=0.001) higher compared to non-inoculated plants (Table 8.21). Within the defoliated plants, the timing of the defoliation event did not significantly (p=0.213 and p=0.014) affect the soluble and insoluble carbohydrate levels, respectively. Levels of soluble carbohydrates in the bark of trees defoliated 2, 4 and 6 weeks prior to inoculation decreased as a result of inoculation, although not significantly (p=0.311) (Table 8.22; Figure 8.19). However, within the defoliated plants, those which were inoculated had significantly (p=0.007) higher levels of insoluble carbohydrate compared to non-inoculated, defoliated plants (Table 8.23; Figure 8.19). Wounding did not significantly (p=0.361 and p=0.027) affect the soluble or insoluble carbohydrate levels, respectively (Table 8.22).

Table 8.20 ANOVA of soluble carbohydrate content (mg/mg FW) in bark of non-defoliated *Eucalyptus globulus* and those trees defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	<0.0001	<0.0001	12.540 (4, 45)	**<0.0001**
Inoculation (2)	<0.0001	<0.0001	2.961 (1, 45)	0.092
1 x 2	<0.0001	<0.0001	0.995 (4, 45)	0.420

Table 8.21 ANOVA of insoluble carbohydrate content (mg/mg FW) in bark of non-defoliated *Eucalyptus globulus* and those trees defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	<0.0001	<0.0001	2.478 (4, 45)	0.057
Inoculation (2)	<0.0001	<0.0001	11.976 (1, 45)	**0.001**
1 x 2	<0.0001	<0.0001	0.852 (4, 45)	0.500

Table 8.22 ANOVA of soluble carbohydrate content (mg/mg FW) in bark of *Eucalyptus globulus* defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	<0.0001	<0.0001	1.571 (3, 37)	0.213
Inoculation (2)	<0.0001	<0.0001	1.053 (1, 37)	0.311
1 x 2	<0.0001	<0.0001	0.498 (3, 37)	0.686

Table 8.23 ANOVA of insoluble carbohydrate content (mg/mg FW) in bark of *Eucalyptus globulus* defoliated to 80% total leaf number 6, 4, 2 and 0 weeks prior to inoculation with *Endothiella eucalypti*. Significant value in bold font.

Effect	MS Effect	MS Error	F (df)	P
Defoliation (1)	<0.0001	<0.0001	4.047 (3, 37)	0.014
Inoculation (2)	<0.0001	<0.0001	8.070 (1, 37)	**0.007**
1 x 2	<0.0001	<0.0001	0.486 (3, 37)	0.694

Table 8.22 ANOVA of (A) soluble and (B) insoluble carbohydrate content (mg/mg FW) in bark of wounded and non-wounded, non-defoliated *Eucalyptus globulus*.

Effect	MS Effect	MS Error	F (df)	P
Wounding (A)	<0.0001	<0.0001	0.915 (1, 10)	0.361
Wounding (B)	<0.0001	<0.0001	6.734 (1, 10)	0.027

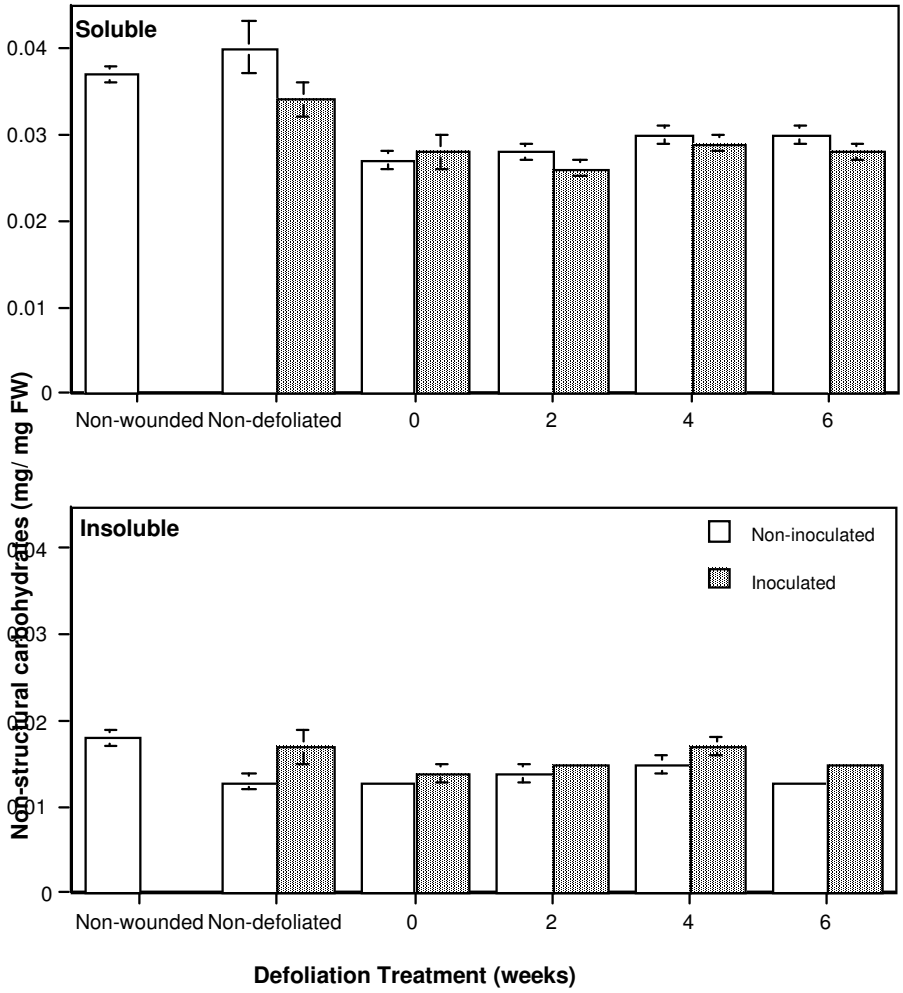


Figure 8.19 Soluble and insoluble non-structural carbohydrate levels (mg/mg FW) in the bark of *Eucalyptus globulus* either non-wounded, non-defoliated or defoliated to 80% of total leaf number 6, 4, 2, and 0 weeks prior to inoculation with *Endothiella eucalypti*. Measurements were taken from non-inoculated and inoculated stems. Bars indicate standard errors of the mean. Where bars are not visible, standard errors are minimal. Missing data indicates non-wounded plants which were not inoculated as this would void treatment.

DISCUSSION

The susceptibility of *E. globulus* to *En. eucalypti* was influenced by both the percentage of the foliage removed in a single defoliation event and the period of time the tree had been exposed to the defoliation stress prior to pathogen challenge. *Eucalyptus globulus* trees were able to withstand removal of a large percentage of leaf area for prolonged periods without seriously compromising its resistance to *En. eucalypti*. It is thought that this ability to withstand high levels of defoliation was due to (i) the rate at which *E. globulus* was able to replace leaf area, (ii) the ability of *E. globulus* to up-regulate

photosynthetic rate in the remaining leaves and (iii) quantity, utilisation and transport of stored carbohydrates.

In the first trial, severe defoliation (100%) was required to induce significantly larger lesions as a result of *En. eucalypti* inoculation, compared to non-defoliated trees. This result has been shown previously in studies on *Populus tremuloides* where the removal of large percentages of leaf area (75% and 100%) was required to sufficiently weaken host defences to the opportunistic pathogen *Cytospora chrysosperma* (Pers.:Fr.) Fr. Hinds (Guyon *et al.*, 1996). This decrease in resistance was linked to the carbohydrate content in the tree roots which was significantly less in those defoliated to 100% compared to trees defoliated to 50% and 75%. In contrast, findings by Paap (2001) reported a decrease in the extent of *Endothiella* colonisation in *Co. calophylla* seedlings defoliated 100% compared to non-defoliated seedlings. In addition, Lawrence (1998) reported that completely defoliated *E. globulus* seedlings did not have significantly larger lesions than non-defoliated seedlings when artificially inoculated with *Endothiella*. The differing results regarding the impact of defoliation on the susceptibility of the host to nonaggressive pathogens may depend on the host species, the severity of the defoliation event and the age of the host at the time of defoliation.

The ability of a tree to re-sprout after a defoliation event is an important response required to maintain the integrity of the host defence system in the constant presence of opportunistic pathogens. In the current study, *E. globulus* regenerated more rapidly after the removal of 90% to 100% of the total leaf area than at lower defoliation rates, with new leaves evident within 7-10 days of the defoliation event. Previously, Old *et al.* (1990) investigated the susceptibility of five tree species, 18-months-old, before and after defoliation to almost 100% and then inoculation with either *Cryphonectria eucalypti* or *Botryosphaeria ribis* under greenhouse conditions in south-eastern Australia. Their results showed that *E. regnans* and *E. delegatensis* were highly susceptible to pathogen invasion following one defoliation event (almost complete defoliation) ten days prior to inoculation. The rate of fungal infection and girdling in these two species warranted harvesting four weeks after inoculation. In comparison, the remaining three species *Co. maculata, E. grandis* and *E. saligna* showed no increase in susceptibility to either fungus, even after two almost total defoliation events (the first defoliation treatment ten days prior to and the second defoliation four weeks after inoculation) (Old *et al.*, 1990). The difference in susceptibility was attributed to the rapid rate of leaf replacement of *E. saligna* and *E. grandis* after defoliation. This

difference in resistance was also correlated with the high levels of stem sugars and starch in *E. grandis* compared to *E. regnans* and the more rapid recovery of starch concentration in *E. grandis* following defoliation (Old *et al.*, 1990).

In the current trial, preliminary investigations of starch accumulation in the bark of *E. globulus* stems did not indicate a significant decrease in levels as a result of defoliation. This may be explained by the patterns of defoliation employed in this trial, whereby trees were kept defoliated prior to inoculation and for three weeks after inoculation, however for the remaining nine weeks of the trial the leaves were allowed to regenerate. As *E. globulus* rapidly replaces foliage lost to defoliation, the starch reserves may have been actively replenished by the time of harvest. In comparison, studies on *Angophora costata,* a tree species in which starch reserves vary seasonally, showed that defoliation significantly reduced starch reserves (Bamber and Humphreys, 1965). The levels of starch returned to those equal to non-defoliated trees within twelve months, however the levels in continuously defoliated trees continued to decline (more slowly than the initial reduction response) until the levels reached zero (Bamber and Humphreys, 1965). As the composition of sugar components within the phloem sap of *E. globulus* stems has been shown to vary according to the time of year and environmental conditions (Pate *et al.*, 1998), it is also possible that the levels of starch varies seasonally. It has been shown previously that the starch granules in the stems of Norway Spruce completely disappear between August and November. The amount of starch in a tree is also dependant on the age and location of the tissues sampled (Viiri *et al.*, 2001). Therefore, these factors need to be taken into account in future when assessing the impact of defoliation on starch levels.

Methods developed by Pate *et al.* (1998) to measure phloem sugars by bleeding phloem sap of *E. globulus* is a fast, effective and non-destructive method of monitoring changes in sugar levels. This method of monitoring phloem sugars was attempted during the current trial, however factors such as tree age and time of year rendered this technique unsuccessful, as sufficient quantities of sap could not be routinely obtained. Investigation of phloem sugar levels in stems of *E. globulus* throughout the year, as well as under difference climatic conditions, are required to determine whether these levels affect the impact of defoliation and disease.

The impact of continuous defoliation to 80% total leaf number on *E. globulus* and its susceptibility to *En. eucalypti* was addressed in the second trial. It was shown that the

trees continuously defoliated for the longest period (6 weeks) had significantly larger lesions compared to those defoliated for a lesser period, or non-defoliated. The length of exposure to defoliation stress has previously been linked to the ability of a plant to recover from pathogen challenge (Schoeneweiss, 1981; Crist and Schoeneweiss, 1975). For example, defoliation beyond the thresh-hold period of three to four weeks has been shown to predispose woody stems to attack by nonagressive canker fungi (Schoeneweiss, 1981). If plants were allowed to refoliate within a certain period, predisposition to canker fungi was reversed and canker formation ceased. However, prolonged exposure to defoliation rendered the plants unable to respond to pathogen attack which resulted in the death of the host (Schoeneweiss, 1981). It is likely that the longer the *E. globulus* were maintained at 80% defoliation, the more rapid the rate of lesion extension in response to inoculation with *En. eucalypti,* ultimately resulting in tree death.

Defoliation significantly increased the rate of photosynthesis in *E. globulus* compared to non-defoliated trees. This change in photosynthetic rate may simply be the result of the increased light intensity reaching each leaf of a defoliated tree or, more likely, a more complex system of photosynthetic compensation due to reduced photosynthetic leaf area. Up-regulation of photosynthetic rate is a widely acknowledged response of plants of various species to a reduction in photosynthetic leaf area (Chen *et al.*, 2001; Tiffin, 2000). Photosynthetic compensation may enhance tree recovery by increasing carbon uptake from the remaining leaves (Chen *et al.*, 2001). In addition, the loss of photosynthetic leaf area may reduce transpiration which reduces the demands on the root system, minimising impairments to fitness (Tiffin, 2000). For example, photosynthetic compensation has been reported in uninfected leaves of peas infected with the leaf pathogen *Erysiphe pisi* (powdery mildew). However, stimulation of photosynthesis also occurs in healthy plants after insect or manual defoliation (Ayres, 1991). This effective adaptation to loss of leaf area may help to explain why *E. globulus* were able to tolerate defoliation of a large proportion of leaf area (100%) demonstrated in the first trial, even in the presence of *En. eucalypti*.

As carbohydrates are a direct product of photosynthesis, a reduction in photosynthetic area via defoliation, results in a reduced carbohydrate content of a plant (Turner *et al.*, 2001). It is this reduction in carbohydrate content that is thought to render the defoliated host more susceptible to nonaggressive pathogens. In the current trial, defoliated *E. globulus* had significantly lower levels of soluble carbohydrates compared

to non-defoliated plants. Previously Old *et al.* (1990) reported that low carbohydrate levels in the stems of defoliated *E. regnans* may be responsible for inhibition of some of the key components involved in the defence response (such as callus formation), which allowed more active invasion by *C. eucalypti*. However, the length of time the *E. globulus* plants were defoliated in the current study did not affect the levels of carbohydrates in the stem. This may be accounted for by the similar increase in photosynthetic rate observed within the defoliated plants. It is also possible that the length of time the *E. globulus* trees were held at 80% defoliation within the current trial (six weeks) was not great enough to further deplete carbohydrate levels. However, it is interesting to note that the trees defoliated for six weeks prior to inoculation did have smaller stem diameters, indicating a reduced growth rate. Therefore, although the defoliated *E. globulus* exhibited an enhanced rate of photosynthesis compared to the non-defoliated plants, the demand for carbohydrates for general growth and the additional production of compounds for defence against *En. eucalypti,* resulted in low carbohydrate levels in defoliated plants.

In conclusion, the removal of a large percentage of total leaf area (100%) or continuous defoliation at high levels (80%) for extended periods (six weeks) was required to significantly decrease the resistance of *E. globulus* to *En. eucalypti*. *Eucalyptus globulus* responds to defoliation by up-regulating photosynthesis in the remaining leaves and rapidly replacing lost foliage. Therefore, the impact of *En. eucalypti* as a pathogen of *E. globulus* in the plantation environment may increase when defoliation is severe and continuous. With the current increase in damage to *E. globulus* foliage caused by insects and fungi (particularly *Mycosphaerella*) within the plantation environment (Maxwell *et al.*, 1998), it is possible that stress induced by defoliation is capable of predisposing trees to attack by pathogens such as *En. eucalypti* in the future.

Chapter 9

General Discussion

Endothiella eucalypti was the most commonly isolated pathogenic fungus associated with trunk and branch cankers of *Eucalyptus globulus* in southwestern Australia. Despite its widespread distribution, the fungus does not appear to have a major impact on the health of plantation-grown *E. globulus.* However, evidence from elsewhere suggests that *En. eucalypti* is capable of causing serious disease or tree death and is considered a threat to *Eucalyptus* plantations in some regions (Gryzenhout *et al.*, 2003; Hardy and Burgess *pers. comm.*; Yuan and Mohammed, 1999, 2000; Wardlaw, 1999). Examples of *En. eucalypti* linked to serious disease outbreaks include *E. smithii* (Gully gum) in South Africa (Gryzenhout *et al.*, 2003), *E. nitens* in Tasmania (Wardlaw, 1999) and *E. dunnii* (Dunn's white gum) in Queensland (Hardy and Burgess *pers. comm.*). Although drought was identified as the key factor in the disease outbreak in Queensland, there is no evidence of an environmental stress to account for the impact of *En. eucalypti* in the other regions. There is also increasing evidence that *En. eucalypti* may be associated with the serious decline in *Corymbia calophylla* in the southwest of WA (Paap, 2001). *Endothiella eucalypti* has been isolated from dying mature *Co. calophylla* trees exhibiting massive cracking and kino exudation. Outbreaks are most severe in disturbed sites, although they do occur in healthy stands. These diseased *Co. calophylla* are often located in small remnant natural bushland areas or on the roadsides in close proximity to *E. globulus* plantations (Paap, 2001). Therefore, the widespread presence of *En. eucalypti* in *E. globulus* plantations and native bushland, as well as evidence for its ability to cause major disease elsewhere, is of concern for the future of plantation forestry in WA.

Host-pathogen interactions

Canker fungi, such as *En. eucalypti,* are generally classified as opportunistic pathogens with limited ability to invade the tissues of healthy trees. However, observations in the field have shown these fungi can be associated with extensive lesions, even in the absence of environmental stress (Gryzenhout *et al.*, 2003; Wardlaw, 1999; Old *et al.*, 1990; Shearer *et al.*, 1987). The nature of the interaction between the canker fungi and the host, which results in infection under some circumstances and not others, is still undefined. However, many of the characteristics of *En. eucalypti* are consistent with those of an endophytic fungus.

Endophytic fungi are able to colonise healthy plant tissue without causing disease (Smith *et al*., 1996b). In their natural environment, they only cause disease on stressed trees and are considered opportunistic pathogens. For example, *Botryosphaeria ribis* and *Sphaeropsis sapinea* have been identified as endophytic species of *Pinus* and *Eucalyptus* in South Africa (Smith *et al.*, 1996a). *Botryosphaeria ribis* has been isolated from latent endophytic infections in eucalypts including *E. globulus.* However, it is also considered one of the most important pathogens of *Eucalyptus* in South Africa and is often particularly pathogenic in stressed trees (Smith *et al.*, 1994). At present, the most commonly identified endophyte in Australia on eucalypt twigs is *Cytospora eucalypticola* (Old and Davison, 2000). In the current field survey and those conducted previously (Yuan and Mohammed, 1997; Davison and Coates, 1991), *En. eucalypti* was observed sporulating abundantly on superficial cankers or dead plant material. In addition, *En. eucalypti* was also widely recovered from bark of *E. globulus* within plantations, with no evidence of serious disease or tree death.

Previous studies have indicated that an entrance point such as an insect wound or split bark was required for invasion by canker fungi (Shearer, 1994). In the current study, *En. eucalypti* was able to invade healthy tissue and cause disease (under controlled conditions) without an entry point such as a wound or crack in the bark on young *E. globulus* stems. It has previously been established that endophytes, such as *Plectosphaera eucalypti,* are able to penetrate the intact host tissue of *E. globulus* (Bettucci and Saravay, 1993). Therefore, there is evidence that *En. eucalypti* exhibits many of the characteristics of an endophyte of *E. globulus*. Opportunistic and endophytic pathogens pose a serious threat to the plantation environment as their constant presence on healthy hosts affords them opportunity to invade at the first sign of host stress or wounding. Further investigations into the processes involved in infection of *E. globulus* with *En. eucalypti* are required.

In the current study, the genotypic diversity within the population of *En. eucalypti* in WA, established by VC analysis, was higher than the asexual population of *Cryphonectria cubensis* in South Africa (van Heerden and Wingfield, 2001). The South African *C. cubensis* population is considered to have been introduced. The origin of *En. eucalypti* in WA is currently unknown. In addition, the teleomorph has not yet been identified in WA. The absence of the *En. eucalypti* teleomorph, is evidence against the hypothesis of a native origin for the fungus in WA (Davison and Tay, 1983) is unsubstantiated. It is unlikely that the surveys conducted during the

current study and those conducted previously (Yuan and Mohammed, 1997; Davison and Coates, 1991) would have failed to isolate the teleomorph if it were present. As the genotypic diversity of an introduced population is generally lower compared to a native population, this further confuses the possible origin of this pathogen. It seems unlikely that the asexual stage of this pathogen was introduced into WA on several occasions, which would account for the level of genotypic diversity recorded in the current study. However, if the pathogen was present in a small number of nurseries which distributed infected stock from throughout Australia to a wide number of centers throughout WA, the potential for a diverse population is high. The question would then be, why was the teleomorph not also distributed throughout the state? In order to establish the origin of *En. eucalypti* in WA, further population studies are required to compare the WA *En. eucalypti* population collected from within *E. globulus* plantations with the isolates causing serious disease in *Co. calophylla* in WA, isolates from the eastern states (mainland), those associated with the outbreak in Tasmania (Yuan and Mohammed, 1999; Wardlaw, 1999) and isolates from South African which have been closely associated genetically with WA isolates. In addition, extensive surveys of native vegetation throughout the southwest of WA need to be conducted to confirm the absence of the *En. eucalypti* teleomorph.

The discovery of *C. cubensis* on the roots and crown of *E. marginata* in southwestern Australia has been the only record of this pathogen in Australia (Davison and Coates, 1991). *Cryphonectria cubensis* is considered one of the most destructive fungal pathogens of *Eucalyptus* trees in plantations and is thought to pose a much greater threat to plantation health in WA at present than *En. eucalypti*. *Cryphonectria cubensis* was not isolated from stems of *E. globulus* during the current study. It is not known whether this pathogen is present on the roots of this species, but this would be unlikely. It is possible that the *C. cubensis* identified on *E. marginata* was an isolated introduction, perhaps limited by environmental conditions. It is interesting to note that according to ISSR-PCR analysis, the *C. cubensis* isolate from WA was most similar to an isolate of the same species collected from an *E. grandis* x *E. urophylla* hybrid in China. Considering this pathogen has not been isolated since its original collection in WA, the hypothesis that the isolate collected from *E. marginata* may have been an isolated introduction, with a possible connection with China, is more likely. Spread of this pathogen into the plantation environment could be disastrous for the industry. However, as it is favoured by high rainfall (2000 - 2400 mm/annum) and temperatures

above 23 °C, a disease epidemic would be confined to regions of suitable climatic conditions (van Heerden and Wingfield, 2001; Conradie *et al.*, 1990). These conditions do not occur in WA.

Observations of *E. nitens* provenances located at the site of a severe disease outbreak in northern Tasmania indicated that trees with rougher bark were more likely to be associated with canker damage than smoother barked trees (Wardlaw, 1999). As discussed in Chapter 4, the fissures which develop in the rough bark, from which the pathogen was often isolated, provide an ideal entrance point for *En. eucalypti* and rough-barked species are therefore more susceptible (Wardlaw, 1999). Pathogenicity studies on some of the isolates collected from the epidemic site in Tasmania indicated that, when wound inoculated, smooth barked *E. nitens* were more susceptible to *En. eucalypti* than rough-barked species. The variation in susceptibility between rough and smooth-barked trees was attributed to the arrangement of phloem parenchyma cells. In rough-barked trees, the arrangement of small thick-walled, closely packed phloem parenchyma cells appeared to provide a physical barrier to the invading pathogen. In comparison, the smooth bark had thin-walled, loosely arranged phloem parenchyma cells which facilitated the invasion of the fungus (Yuan and Mohammed, 2001). Rough bark was not considered a response to infection, more a characteristic which is prevalent in certain provenances (Yuan and Mohammed, 2001). In the absence of a wound, the fissures within the rough bark may harbour the pathogen until environmental conditions are suitable for invasion. Other factors such as gaps in periderm layers may also present access routes for the pathogen, however this requires further investigation.

The results of the current study and those undertaken previously (van Heerden and Wingfield, 2001) indicate that host provenances may respond differently to infection with canker-causing fungi, with some provenances being more susceptible than others. In addition, a significant link between site and host provenance was reported, both in the current study and that of van Heerden and Wingfield (2001). The development of disease was dependent upon the environmental conditions of the site, rather than on the pathogenicity of the fungal isolate. Therefore, screening for host resistance to canker-causing fungi should be conducted at sites where the provenance/clone of interest is to be propagated. Although previous studies have questioned the validity of artificial inoculation techniques in selecting provenances for use in the field (Yuan and Mohammed, 2000), the current study and previous studies on eucalypts (van Heerden

and Wingfield, 2001) have indicated that artificial inoculation trials provide an accurate indication of host susceptibility.

Role of environmental stress in disease epidemics

In order to explain the patterns of pathogenicity exhibited by *En. eucalypti*, environmental stress has been identified as a key factor associated with serious disease (Yuan and Mohammed, 2000). For example, the disease outbreak associated with *En. eucalypti* on *E. dunnii* in Queensland was linked to stress induced by prolonged drought (Hardy and Burgess *pers. comm.*). In comparison, the disease epidemic associated with *C. eucalypti* in Tasmania was located at one of the most productive *E. nitens* sites and trees were reportedly growing vigorously at the time of infection. It was concluded from this outbreak that host vigour did not influence the establishment of stem cankers at this site (Wardlaw, 1999). The role of defoliation stress was addressed in the current study and it was shown that severe defoliation was required to significantly alter the susceptibility of *E. globulus* to *En. eucalypti*. At present the evidence for the role of stress in facilitating outbreaks of canker disease is incomplete, however it does appear that severe stress disrupts the balance between host and pathogen resulting in serious disease and tree death.

If environmental stress is required to allow canker fungi such as *En. eucalypti* to cause serious disease (girdling cankers often resulting in tree death) in eucalypts, the adaptation of the host to environmental conditions may affect its degree of susceptibility to the pathogen. In the current study, *E. globulus* quickly adapted to continuous defoliation by replacing lost foliage whilst up-regulating photosynthesis in the remaining leaves to partially compensate for the reduction in leaf area. The ability of *E. globulus* to maintain this elevated level of activity requires further study. However, it does provide further evidence for the hypothesis that severe host stress may lower eucalypt host resistance to *En. eucalypti*. From an industry perspective, this is an important aspect to consider when selecting a clone/provenances to suit site conditions or choosing appropriate sites if suitable provenances/clones are not available for more adverse sites. In addition, minimising the damage to *E. globulus* foliage by insects and foliar diseases such as MLD may help maintain host resistance to canker-causing fungi such as *En. eucalypti*.

Severe drought stress has been shown to increase host susceptibility to canker-causing fungi both in the glasshouse (Blodgett *et al.*, 1997; Maxwell *et al.*, 1997; McIntyre *et*

al., 1996, Guyon *et al*., 1996; Jacobi, 1992; Appel and Stipes, 1984; Schoeneweiss, 1981; Crist and Schoeneweiss, 1975) and the field (Hardy and Burgess *pers.comm.*). However, the reverse has also been shown. For example, colonisation of one-year-old *E. grandis* plants by *C. cubensis* following artificial inoculation was inhibited by drought stress (Swart *et al.*, 1992). Similarly, neither drought nor waterlogging had a significant effect on the extent of disease in stems of one-year-old *E. globulus* seedlings artificially inoculated with *Endothiella* (Tovar, 1998). More recently, cankers caused by an *Endothiella* were restricted in excised *Co. calophylla* stem material allowed to dry for two days at 20 °C compared to healthy seedlings (Paap, 2001). These results suggest that the impact of drought itself does not facilitate canker development as stem moisture levels are low and perhaps not conducive to extensive colonisation by the pathogen. However, it appears likely that a period of wet conditions following a dry period may increase stem moisture thus facilitating fungal colonisation, triggering a disease outbreak such as was seen in Queensland (Hardy and Burgess *pers. comm.*). Unlike *C. cubensis,* which is restricted to warm, high rainfall areas, *En. eucalypti* has a wider distribution and is therefore more likely to be associated with a drought stressed host. This is particularly true of WA plantations as the high demand for land to support future plantations extends into more marginal areas.

It has been suggested that drought tolerant plants may be less susceptible to canker causing fungi (Swart *et al.,* 1992). *Eucalyptus globulus* is not well adapted to cope with drought and, if it survives the drought-stress, may be a prime target for a drought stress-induced epidemic. This is of particular concern as the timing of the period of water stress in relation to challenge by a pathogen does not appear to affect the severity of the resultant disease (Paoletti *et al.*, 2001). For example, previous studies on *Pinus halepensis* (Aleppo pine) seedlings have shown that the host was more susceptible to the canker fungus, *S. sapinea,* regardless of whether the stress occurred before or after the fungal infection (Paoletti *et al*., 2001). Further studies investigating the effect of drought stress on the *E. globulus* host physiology and the degree of drought stress required to significantly weaken the host defences are required. In addition, selection of more drought tolerant *E. globulus* provenances is required when planting in dryer areas of WA. Drought stress was not investigated in this thesis as the southwest of WA is generally well watered (Mattinson *pers. comm.*).

Control strategies

The variability in pathogenicity amongst *En. eucalypti* isolates associated with *E. globulus* in WA, together with the host-environment interactions, makes control of this pathogen complex. To date, control of canker-causing fungi in eucalypt plantations has been limited to the selection of more resistant provenances or clones (Gryzenhout *et al.*, 2003; van Heerden and Wingfield, 2002; Old and Davison, 2000). However, as *E. globulus* is very difficult to clone, the results of this thesis suggest that the selection of more resistant provenances in association with environments suitable for optimal growth of that particular provenance, should limit disease outbreaks caused by *En. eucalypti*. In addition to careful site selection, the development of molecular markers linked to host tolerance to disease, established in pathogenicity trials, is currently underway (van Heerden and Wingfield, 2001). The application of this technology will provide a useful method for future selection of plant stock.

Another potential avenue for disease control exists through the use of dsRNA mediated-hypovirulence which has previously been successful in reducing pathogen virulence (Robin *et al*., 2000). Infection with hypovirulent isolates of *C. parasitica,* the causal agent of chestnut blight, resulted in superficial cankers, whereas virus-free isolates caused lethal cankers (Robin *et al*., 2000). Cryphonectria hypoviruses (CHV) are cytoplasmic double-stranded RNA viruses that move into conidia and can be transmitted from an infected isolate to a virus-free isolate through hyphal anastomosis (Robin *et al.*, 2000). The rate of transmission is negatively correlated with the number of vegetative incompatibility (*vic*) genes that differ between mycelia that anastomose (Robin *et al*., 2000). Therefore, biological control using hypovirulence depends on the ease of dsDNA spread, which is favoured by isolates within the same VC group. For example, in South Africa where there is low genetic diversity within the population of *C. cubensis* it could be possible to reduce pathogen virulence through dsRNA mediated-hypovirulence (van Heerden and Wingfield, 2001; Allemann *et al.*, 1999). Although further research is required into the diversity of the WA population of *En. eucalypti*, the use of dsRNA mediated-hypovirulence in reducing pathogen virulence would not be a useful strategy in reducing the impact of this pathogen in WA, as the current study showed a high level of genotypic diversity with 25 VC groups from 26 isolates.

As plantations are even-aged monocultures they are more susceptible to disease than native forests, where epidemics are restricted due to the age structure and the diversity of the plant community. Although there is no immediate disease threat to *E. globulus*

plantation health in WA, the introduction of more virulent pathogens such as *C. cubensis* from native forest areas or overseas could be devastating to the industry.

Global trade agreements and the removal of embargoes and tariffs have facilitated the movement of forestry products around the world, thus increasing the risk of pathogen introductions (Palm, 1999). This is an area of great concern, as an introduced pathogen may spread undetected until a significant area has been affected, by which time it may be too widespread to eradicate (Walker, 1987). In addition, the introduction of the *En. eucalypti* teleomorph would significantly effect the population diversity of the currently asexual WA population. Although there is no evidence to suggest that, if introduced into WA, the teleomorph from the eastern states would necessarily be more pathogenic than isolates of *En. eucalypti* already present (Yuan and Mohammed, 2000), an increase in the diversity of the *C. eucalypti* population would further complicate control strategies. Therefore, strict quarantine measures are required to ensure that plant material entering WA from overseas or the eastern states does not serve as a source of exotic pests and diseases. Of particular importance is the introduction of plant material for the screening and development of new genetic lines. Importation of this type of material requires thorough inspection and quarantine, detailed enough to detect the presence of endophytes, prior to release.

The ability of *En. eucalypti* to exist on healthy stems within a plantation environment provides the ideal opportunity for pathogen dissemination and increases the potential for disease development. As the mechanisms involved in *En. eucalypti* infection and the role of environmental stress on subsequent disease development are currently poorly understood, records of the presence of this fungus are as important as records of the outbreaks of disease in the management of eucalypt plantations. Therefore, identification of and monitoring the extent of *En. eucalypti* present in the WA plantation environment needs to be an integral part of plantation health surveys at various times of the year, along with details of current environmental stresses, such as climatic conditions, insect attack and nutrient status. In addition, the establishment and assessment of permanent monitoring plots in which pathogen presence, disease levels, pathogen introductions, identification of factors influencing the outbreaks of disease, such as changes in environmental conditions, and the susceptibility of *E. globulus* provenances to this disease will further add to the knowledge in managing disease within the plantation estate.

The results of the survey conducted in Chapter 2 clearly indicate that regions, such as Esperance, with a younger plantation estate, have a much lower incidence of *En. eucalypti* compared to regions with a longer history of *E. globulus* plantations, such as Bunbury. It is likely then, that over time the level of disease will continue to increase as the WA plantation estate ages. Therefore, the development of strategies to monitor and restrict the spread of this pathogen are required to ensure the health and productivity of plantation grown eucalypts in WA. Management options which may serve to limit the future spread of *En. eucalypti* in the plantation environment include (i) increasing the distance between trees providing more conducive environmental conditions for the healthy growth of the host (reducing possible nutrient and water stress), and (ii) limiting the amount of dead organic matter which remains following coppicing which will reduce the build-up of canker fungi within second rotation plantations, thereby reducing the source of future inoculum.

Future directions

Currently, methods for assessment of fungal pathogenicity and host susceptibility rely on artificial inoculations and measurement of fungal colonisation of the host. If the use of more resistant provenances/clones is currently the only method of control for canker-causing fungi within plantations, development of more accurate assessments of host susceptibility/fungal pathogenicity, in addition to fungal colonisation, are required. For example, van Zyl and Wingfield, (1998) have shown that a *Eucalyptus* clone previously classified as highly susceptible to *C. cubensis* produced significantly more ethylene than a disease-resistant clone. In addition, *Eucalyptus* clones infected with a hypovirulent isolate of *C. cubensis* produced significantly lower levels of ethylene than was induced by a virulent isolate (van Zyl and Wingfield, 1998). As discussed in Chapter 5, the ability to close wounds has also been shown to be a good indicator of the resistance of *E. grandis* clones to *C. cubensis* (van Zyl and Wingfield, 1999). The levels of plant phenolics produced both in response to infection or wounding, or produced constitutively can also be used as an indication of host susceptibility. As illustrated in Chapter 7, the stem of a more resistant *E. globulus* host had higher constitutive levels of soluble and bound phenolics in the region above the area of challenge. In addition, studies on *E. nitens* have shown that the degree of phenolic accumulation was related to the aggressiveness of the fungi, with aggressive fungi eliciting a greater accumulation of phenolics in advance of infection compared to weakly aggressive fungi or sterile inoculations (Barry *et al.*, 2002). The greater

phenolic production associated with the presence of a more aggressive fungus was attributed to the continual challenge of the host xylem cells in response to pathogen challenge constantly eliciting host defence mechanisms (Barry *et al*., 2002). Previously, studies by Cahill *et al.* (1993) linked phenolic accumulation with the resistance of *E. marginata* to *Phytophthora cinnamomi*. Other studies with *E. marginata* showed that those clones more resistant to *P. cinnamomi* infection had both higher levels of phenolics and other defence enzymes of the phenylpropanoid pathway on a constitutive level and in response to pathogen challenge (Jackson, 1997). Analysis of soluble and bound phenolic levels using spectrophotometric analysis is a relatively fast, inexpensive and reliable assessment of host defence mechanisms that relate directly to host resistance. Therefore, development of a bioassay to rapidly assess the resistance of a host to canker-causing fungi, such as measurements of plant phenolic levels, ethylene production or rate of wound closure, may be useful tools in addition to fungal colonisation of the host, in screening for more resistant provenances or as an indication of isolate aggressiveness. Further understanding of the events occurring at the host-pathogen interface will not only aid in provenance selection, but enhance our understanding of the infection process of *En. eucalypti*.

The identification and use of non-pathogenic isolates has previously been effective in stimulating host defence responses without causing disease. This enables the host to respond more rapidly to a future attack by a more aggressive pathogen, thus limiting the impact of the aggressive pathogen (Olivain and Alabouvette, 1997; Fink *et al.* 1991). A susceptible host may have the mechanisms required for resistance, however it is unable to activate these responses in time nor with sufficient intensity to effectively restrict pathogen invasion (Cahill *et al.*, 1993). The more rapid the host response to pathogen challenge, the more likely pathogen ingress will be halted. Therefore, induction of the host defence mechanisms by a non-pathogenic isolate protects (to some extent) the host against more pathogenic isolates in the future. The use of this method of control requires intensive screening of all possible *E. globulus* hosts both in the glasshouse and the field for susceptibility to a range of *En. eucalypti* isolates. As discussed in Chapter 4, the interaction between the host, environment and the pathogenicity of the *En. eucalypti* isolate influences the development of disease and highlights the importance of testing for pathogenicity under glasshouse and field conditions. If ideal isolates were identified, dispersal within the plantation environment would be relatively easy to facilitate. However, the likelihood of a non-pathogenic isolate of *En. eucalypti*

invading a healthy host to induce the host defence responses prior to a more pathogenic isolate is probably remote, but warrants further investigation.

Conclusions

Endothiella eucalypti was widespread in two- and three-year-old *E. globulus* plantations in the southwestern Australia. The effect of *En. eucalypti* on *E. globulus* varied according to the fungal isolate, host provenance and environmental conditions, however some isolates were more pathogenic than others. At present the strategies for limiting disease caused by *En. eucalypti* within *E. globulus* plantations in southwestern Australia include:

(i) provenance/clone selection for suitability to prevailing environmental conditions,

(ii) careful site selection and regular monitoring of tree health/ levels of disease;

(iii) establishment and regular assessment of permanent monitoring plots;

(iv) management of residue in second rotations; and

(v) strict quarantine procedures to limit the import of potential pathogens.

Although, *En. eucalypti* does not currently pose a major threat to the health of *E. globulus* plantations in WA, severe environmental stress such as drought and defoliation increases the susceptibility of the host to this pathogen.

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Appendix

Table 4.1 Labeling of trees exposed to each of the 30 fungal isolates and the control treatment for each provenance at each location.

Site:
Provenance:

Tree Number	Row 1 Isolate #	Row 2 Isolate #	Row 3 Isolate #	Row 4 Isolate #	Row 5 Isolate #
1	20	14	10	21	15
2	6	21	3	24	30
3	15	22	21	12	24
4	8	15	22	10	17
5	14	7	12	9	8
6	30	31	14	18	23
7	10	16	11	31	14
8	24	9	23	4	9
9	27	19	4	3	5
10	5	8	31	30	11
11	19	2	9	11	25
12	21	26	2	29	26
13	31	27	25	14	3
14	7	11	19	15	21
15	13	18	27	5	22
16	11	5	30	2	16
17	3	3	15	7	4
18	25	30	5	28	7
19	12	24	7	27	29
20	4	29	8	1	31
21	22	23	16	13	19
22	29	12	29	17	6
23	28	25	1	6	1
24	26	28	24	25	2
25	9	4	28	26	20
26	18	1	13	8	18
27	1	10	20	20	27
28	2	17	17	23	12
29	16	20	26	19	28
30	17	13	18	16	13
31	23	6	6	22	10

Table 7.1 ANOVA of Soluble peroxidase (nkat mg protein^{-1}) for all four *Eucalyptus globulus* stem segments.

Effect	Rao's R (df 1, 2)	P
Copper (1)	0.88 (4, 30)	0.487
Eucalyptus globulus (2)	2.28 (4, 30)	0.084
Inoculation (3)	0.53 (4, 30)	0.714
1 x 2	0.88 (4, 30)	0.488
1 x 3	0.35 (4, 30)	0.844
2 x 3	1.08 (4, 30)	0.385
1 x 2 x 3	1.57 (4, 30)	0.208

Table 7.2 ANOVA of Static peroxidase (nkat mg protein^{-1}) for all four *Eucalyptus globulus* stem segments.

Effect	Rao's R (df 4, 29)	P
Copper (1)	0.70	0.59
Eucalyptus globulus (2)	0.89	0.48
Inoculation (3)	3.31	0.02
1 x 2	0.23	0.92
1 x 3	0.78	0.55
2 x 3	2.02	0.12
1 x 2 x 3	0.11	0.98

Table 7.3 ANOVA of Ionic peroxidase (pkat mg protein^{-1}) for all four *Eucalyptus globulus* stem segments.

Effect	Rao's R (df 4, 28)	P
Copper (1)	0.42	0.79
Eucalyptus globulus (2)	2.67	0.053
Inoculation (3)	3.15	0.029
1 x 2	0.65	0.63
1 x 3	1.15	0.35
2 x 3	1.60	0.20
1 x 2 x 3	0.17	0.95

Table 7.4 ANOVA of Covalent peroxidase (pkat mg protein^{-1}) for all four *Eucalyptus globulus* stem segments.

Effect	Rao's R (df 4, 27)	P
Copper (1)	0.47	0.75
Eucalyptus globulus (2)	3.26	0.026
Inoculation (3)	2.66	0.054
1 x 2	0.31	0.87
1 x 3	0.66	0.62
2 x 3	1.35	0.27
1 x 2 x 3	0.86	0.49

Table 7.5 ANOVA of bound phenolics (mM mg protein^{-1}) for all segments of Cu-*Eucalyptus globulus* stems.

Effect	Rao's R (df 4, 15)	P
Eucalyptus globulus (1)	1.914	0.160
Inoculation (2)	2.303	0.106
1 x 2	0.317	0.862